REMARKS/ARGUMENTS

The application has been amended. In particular, the description of Figure 1 in the Brief Description of the Drawings has been amended to include reference to the appropriate sequence identifiers. Also, the abstract has been replaced. The claims have also been amended. Claims 7 and 8 have been canceled. Subject matter from these claims has been incorporated into new claims 42, 43 and 44. New claims 42-49 are presented herewith.

Objections to the Specification

The Examiner has objected to the specification for not providing the corresponding sequence identifiers for the sequences in Figures 1A-1B. The Examiner has also objected to the form and length of the abstract of the disclosure. Each of these objections has been addressed in the amendments presented herewith.

Objections to the Claims

The Examiner has objected to claims 7-8 due to the recitation of "Upf1p", "eRF1", "eRF3", "Upf3p" and "Upf2p". The Examiner indicates that abbreviations should not be used in the claims without at least once reciting the entire phrase for which the abbreviation is used unless obvious and/or commonly used in the art.

These objections will be addressed in regard to new claims 42-44, which incorporate subject matter from claims 7-8, now canceled.

Claim 42 recites the entire phrases for which the abbreviations "eRF1" and "eRF3" are used. In particular, eRF1 and eRF3 are used as abbreviations for eukaryotic Release Factors 1 and 3, respectively. Moreover, claims 42, 43 and 44 recite a eukaryotic Upf1 protein, a eukaryotic Upf2 protein or a eukaryotic Upf3 protein, respectively. It is commonly known that

these Upf proteins are polysome-associated proteins first identified in genetic screens in *Saccharomyces cerevisiae*, and that they directly mediate the process of nonsense-mediated mRNA decay. Since these abbreviations are commonly used in the art, Applicants submit that no correction is necessary with regard to the recitation of "Upf1p", "Upf2p" and "Upf3p".

Claim Rejections- 35 U.S.C. § 112, Second Paragraph

The Examiner has rejected claim 7 under 35 U.S.C. § 112, Second Paragraph due to the recitation of "MTT1", which is alleged as being indefinite. In particular, it is unclear to the Examiner whether the modulator of translation termination recited in the claims is a helicase B or any group I helicase.

This rejection has been obviated by the language of new claim 42.

Claim Rejections- 35 U.S.C. § 112, First Paragraph

The Examiner has rejected claims 7 and 8 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. In particular, the Examiner alleges that the genus of polypeptides required to make the multiprotein complex is a large, structurally variable genus, and that all of the components of the multiprotein complex are not adequately described. In this regard, the Examiner states the following:

> **"While a sufficient written description of a genus of polypeptides may be achieved by a recitation of a representative number of polypeptides defined by their amino acid sequence or a recitation of structural features common to members of the genus, which features constitute a substantial portion of the genus, in the instant case, there is no structural feature which is**

representative of all the members of the genus of proteins recited in the claims."

The Examiner also states that, even if all of the components of the multiprotein complex were adequately described, it is unclear whether a combination of proteins from different sources when assembled would have the desired activity, i.e., modulation of peptidyl transferase activity.

The Examiner has further rejected claims 7 and 8 under 35 U.S.C. § 112, first paragraph, alleging that the scope of these claims was not commensurate with the enablement provided in regard to the large number of helicase B proteins, Upf proteins, and peptidyl eukaryotic release factors of unknown structure required in the complex. The Examiner does, however, indicate that the specification is enabling for a multiprotein complex comprising *S. cerevisiae* helicase B, *S. cerevisiae* eRF1, *S. cerevisiae* eRF3, *S. cerevisiae* Upf1p, *S. cerevisiae* Upf2p and *S. cerevisiae* Upf3p.

Applicants respectfully traverse these rejections for the reasons set forth below. These rejections will be addressed in regard to new claims 42-44, which include subject matter from claims 7 and 8, now canceled.

The present invention is directed to a multiprotein complex that is effective to modulate peptidly transferase activity during translation. As recited in new claim 42, this complex includes a eukaryotic Modulator of Translation Termination protein (Mtt1p, also referred to as helicase B), a eukaryotic Upf1 protein, a peptidyl eukaryotic release factor 1 (eRF1) and a peptidyl eukaryotic release factor 3 (eRF3). Claim 42 also recites that the components of the complex are from the same eukaryotic source. The complex can further include a eukaryotic Upf2 protein (claim 43) or a eukaryotic Upf3 protein (claim 44).

As recognized by the Examiner, Applicants have demonstrated an actual reduction to practice of an isolated complex including components from the yeast *S. cerevisiae*. Yeast is a

widely accepted model of essential functions in eukaryotic cells. Such essential functions include protein synthesis and its termination.

However, the present invention is not limited to an isolated multiprotein complex of *S. cerevisiae* components. In view of Applicants' disclosure and the common knowledge known to those of ordinary skill in the art, such a skilled artisan would have immediately recognized that Applicants were also in possession of an isolated multiprotein complex containing homologs of the yeast components, the complex originating from a source other than yeast.

As the Examiner is aware, according to the Written Description Guidelines, the analysis as to whether Applicants were in possession of the claimed invention must be conducted from the standpoint of one skilled in the art. Moreover, according to these Guidelines, Applicants need not disclose in detail that which would be conventional or well known to one of skill in the art.

The claims of the present invention clearly recite a eukaryotic eRF1 and a eukaryotic eRF3 as members of the protein complex. The amino acid sequences of various eukaryotic members of the eRF1 family and the eRF3 family were well known to one of skill in the art at the time the application was filed. See Biochem. Cell Biol. (1995) 73:1079-1086, which is attached herewith as Exhibit A. For example, the sequences of eRF1 proteins from at least yeast, rabbit, human and frog were known. Moreover, the sequences of eRF3 from at least yeast, human and frog were also known. It was also known that these eRF1 and eRF3 proteins were involved in termination of ribosome-dependent protein synthesis.

Regarding a eukaryotic Upf1p, Applicants have provided the yeast amino acid sequence in FIG 1A-1B of the present application. In addition to yeast Upf1p, a human homolog of Upf1p had already been isolated, sequenced and found to be functional in enhancing translation termination. This is described in the application (page 3, lines 25-27; page 4, lines 1-3), and also in PNAS USA (1996) 93: 10928-10932, which is attached herewith as Exhibit B.

Applicants have also provided a recitation of structural features of Upf1 and Upf1-like proteins that result in their function in modulating translation termination (See FIG. 1A-1B, for example). These structural features are disclosed as being present in yeast Upf1, and in the human homolog of Upf1, as well (see page 58, lines 1-2).

With respect to eukaryotic Upf2p and Upf3p, at the time the application was filed, these proteins had already been isolated from the yeast *Saccharomyces cerevisiae*, and their sequences were known. See, for example, Cui, et al. (1995) Genes Dev. 9, 423-436 and Lee and Culbertson (1995) PNAS USA 92, 10354-10358, which are attached herewith as Exhibits C and D, respectively.

Because yeast is a widely accepted model of essential functions in eukaryotic cells, one of ordinary skill in the art would anticipate or foresee that human orthologues of Upf2p and Upf3p exist, and that they are part of a multiprotein complex that functions to maintain the fidelity of the translation process and mRNA turnover (see page 1, lines 15-19). On the basis of these facts alone, one of skill in the art would have recognized that Applicants were in possession of a multiprotein complex including a eukaryotic Upf2p (claim 43) or a eukaryotic Upf3p (claim 44).

As would be expected, numerous eukaryotic Upf2p and Upf3p orthologues have subsequently been identified and sequenced, including human homologues. These orthologues have a high degree of structural and functional homology with their yeast counterparts. The human homologues of Upf2p and Upf3p (including two Upf3p isoforms) have been found to function in both translation termination and nonsense-mediated mRNA decay.

Regarding a eukaryotic Modulator of Translation Termination protein (Mtt1p, also referred to as Helicase B), this protein was isolated and sequenced from the yeast *Saccharomyces cerivisiae*. The yeast amino acid sequence is provided in FIG. 1A-1B of the present application.

Furthermore, Applicants' invention contemplated, that mtt1p had a human counterpart, which was a component of the complex (see page 5, lines 18-25).

As described above, Applicants have also provided a recitation of structural features common to Upf1 and Upf1-like proteins that result in their function in modulating translation termination. These proteins include eukaryotic Mtt1p. As shown in FIG. 1A-1B, and as recited in new claim 45, a eukaryotic Mtt1p should comprise at least one of the following motifs to be effective in modulating the fidelity of translation termination: GppGTKTxT-X(n), riLxcaSNxAvDxl-X(n), vviDExxQaxxxxxiPi-X(n), xxilaGDxxQLp-X(n), lxxSLFerv-X(n), LxxQYRMhpxisefpxYxgxL-X(n), IgvitPYxxQvxxl-X(n), vevxtVDxFQGreKdxlilScVR-X(n) and iGFLxdxRRINValTRak. This shows that Applicants were in possession of the necessary structural features of eukaryotic Mtt1 proteins that result in its function in modulating translation termination.

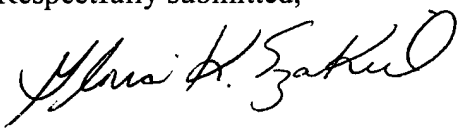
Applicants submit that the disclosure of the present application, when taken together with what was commonly known in the art, adequately describes all components of the multiprotein complex. In particular, one of skill in the art would have understood the inventors to be in possession of the claimed invention.

For similar reasons to those above, Applicants also submit that the scope of the new claims is commensurate with the enablement provided. In particular, Applicants have provided a working example of an isolated multiprotein complex in *Saccharomyces cerevisiae*. Moreover, Applicants have provided sufficient direction in regard to an isolated multiprotein complex including proteins originating from a source other than yeast (e.g., human). Also, the state of prior art was such that sequences of various members of the recited genuses were known. Based upon a review of these known sequences, a high degree of structural homology and functional homology is shared among members of each of the genuses. Thus, in the instant case, the art teaches that the proteins in each genus share a high degree of structural homology, and that they do necessarily share the same function. This is not surprising, given the importance of

maintaining the fidelity of translation termination. When these facts are taken into account, one of ordinary skill in the art would be able to practice the invention as claimed.

In view of the amendments and remarks above, Applicants respectfully request that the 35 U.S.C. § 112, first paragraph be withdrawn. Applicants submit that the present application is in condition for allowance. Entry of this amendment, reconsideration and favorable action are respectfully solicited. Should the Examiner have any questions regarding this response or wish to discuss this matter in further detail, she is invited to contact Applicant's undersigned agent at the telephone number set forth below.

Respectfully submitted,



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Frontiers in translation

An International Conference on the Structure and Function of the Ribosome

Aux frontières de la traduction

Une conférence internationale sur la structure et le fonctionnement du tibosome

Termination of translation in eukaryotes

Lev L. Kisselev and Lyudmila Yu. Frolova

Abstract: Termination of translation is governed in ribosomes by polypeptide chain release factors (pRF and eRF in prokaryotes and eukaryotes, respectively). In prokaryotes, three pRF have been indentified and sequenced, while in eukaryotes, only a single eRF has been identified to date. Recently, we have characterized a highly conserved protein family called eRF1. At least, human and *Xenopus laevis* proteins from this family are active as eRFs in the in vitro assay with any of the three stop codons. No structural similarity has been revealed between any of the three pRFs and eRF1 family. Furthermore, GTP-binding motifs have not been revealed, although translation termination in eukaryotes is a GTP-dependent process. We have demonstrated that in eukaryotes a second eRF exists in addition to eRF1, called eRF3. The eRF3 family has two features in common: presence of GTP-binding motifs and high conservation of the C-terminal domain structure. The C-terminal domain of the *X. laevis* eRF3 has no RF activity although it stimulates the eRF1 activity considerably at low concentration of the stop codons, conferring GTP dependence to the termination reaction. Without eRF3, the eRF1 activity is entirely GTP independent. Some features of *X. laevis* eRF3 (C-terminal domain) resemble those of pRF3. The newly identified eRF1 and eRF3 are structurally conserved and distinct from the respective pRF1/2 and pRF3 proteins, pointing to the possibility of different evolution of translation termination machinery in prokaryotes and eukaryotes. Bipartition of the translation termination apparatus probably provides high rate and accuracy of translation termination.

Key words: higher eukaryotic polypeptide chain release factors, translation termination, protein biosynthesis.

Résumé : La fin de la traduction est contrôlée par des facteurs de libération du polypeptide (les pRF procaryotes et les eRF eucaryotes) se fixant sur les ribosomes. Chez les procaryotes, trois pRF ont été identifiés et séquencés, alors que chez les eucaryotes, un seul eRF a été identifié. Récemment, nous avons caractérisé une famille de protéines très conservées, les eRF-1. In vitro, les eRF-1 humaines et de *Xenopus laevis* reconnaissent les trois codons de terminaison. Il n'y a pas de similitude structurale entre les eRF-1 et les trois pRF. De plus, aucun motif de liaison du GTP n'y a été mis en évidence, même si la fin de la traduction chez les eucaryotes dépend du GTP. Nous avons démontré que, en plus des eRF-1, il y a une deuxième famille d'eRF chez les eucaryotes, les eRF-3. Deux caractéristiques sont communes aux eRF-3 : la présence de motifs de liaison du GTP et la structure très bien conservée du domaine C-terminal. Le domaine C-terminal de l'eRF-3 de *X. laevis* n'agit pas comme un RF, mais il stimule fortement l'activité de l'eRF-1 lorsqu'il y a peu de codons de terminaison, ce qui rend la fin de la traduction dépendante du GTP. En absence de l'eRF-3, l'activité de l'eRF-1 est totalement indépendante du GTP. Le domaine C-terminal de l'eRF-3 de *X. laevis* ressemble à celui du pRF-3. Les eRF-1 et eRF-3 récemment identifiés ont une structure conservée, différente de celle des protéines pRF-1/2 et pRF-3, ce qui indiquerait une divergence évolutive entre le système de terminaison de la traduction des procaryotes et celui des eucaryotes. Cette divergence leur aurait assuré une vitesse élevée et une grande précision.

Mots clés : facteurs de libération du polypeptide des eucaryotes supérieurs, terminaison de la traduction, synthèse protéique.

[Traduit par la rédaction]

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Abbreviations: eRF, eukaryotic polypeptide chain release factor; pRF, prokaryotic peptide chain release factor; SDS–PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; WRS, tryptophanyl-tRNA synthetase (EC 6.1.1.2); kDa, kilodalton; 2D, two dimensional; kb, kilobase(s); Tris, tris(hydroxymethyl)aminomethane; GTP, guanosine 5′-triphosphate.

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Introduction

Protein synthesis in all organisms is arranged in a similar way: it is composed of two major steps: recognition (aminoacylation of tRNAs catalyzed by aminoacyl-tRNA synthetases in cytoplasm) and translation (initiation, elongation, and termination of polypeptide synthesis in ribosomes). However, within this general frame, many features are distinct between prokaryotes and eukaryotes. Two differences are most remarkable and evident. First, most of the components of the eukaryotic protein-synthesizing machinery exhibit higher molecular masses than do the respective components in prokaryotes. For example, eukaryotic ribosomal proteins (Wool 1986) and aminoacyl-tRNA synthetases (Kisselev and Wolfson 1994) are at average much heavier than their prokaryotic counterparts. However, these differences are probably not related to the structure of catalytic or binding centers, but rather to more sophisticated regulatory devices in eukaryotes. Second, there is an increase in the total number of proteins involved in the translation process in eukaryotes. For instance, eukaryotic initiation and elongation factors are more numerous than in prokaryotes (Wittmann 1986; Wool 1986). It is assumed that it reflects the fine tuning of translation in eukaryotes, especially in higher organisms, rather than profound alteration of the catalytic mechanisms.

Contrary to this general pattern, three polypeptide chain release factors (RFs) are known at present in prokaryotes and only a single RF has been so far described for mammalian species (reviewed in Caskey 1980; Craigen et al. 1990). In this latter case, either a single eukaryotic RF (eRF) fulfills the functions of prokaryotic RFs (pRF1, pRF2, and pRF3) or the translation termination in eukaryotes is similar to that in prokaryotes but some of the eRFs have not yet been identified.

A single eRF involved in termination of ribosome-dependent protein synthesis has been identified in reticulocyte lysates and partially purified (Beaudet and Caskey 1971; Goldstein et al. 1970; Konecki et al. 1977; Caskey et al. 1974). It recognizes the stop codons UAA, UAG, and UGA, requires GTP for activity, and consists of two subunits (Beaudet and Caskey 1971; Caskey et al. 1974). Rabbit eRF cDNA has been cloned and sequenced and attempts have been made to deduce the eRF amino acid sequence (Lee et al. 1990). This eRF protein possessed low similarity with bacterial tryptophanyl-tRNA synthetases (WRS) (Lee et al. 1990) and nearly 90% homology with mammalian WRS (Garret et al. 1991; Frolova et al. 1991). For these reasons, it has been suggested (Frolova et al. 1991) and proved (Frolova et al. 1993*a*, 1993*b*) that eRF is in fact rabbit WRS.

Purification of the rabbit eRF1

Because the main source of misassignment of eRF was due to traces of WRS in the eRF preparation (Frolova et al. 1993*b*), it was necessary to improve the protocol of eRF purification. Several purification steps were used to achieve the goal. After the last purification step on the MonoQ column, the protein fractions showing the highest eRF activity yielded a predominant band on SDS–PAGE corresponding with the protein with the mass of ~50 kDa (Frolova et al. 1994), which was lower than that determined previously (54 kDa) for the partially purified rabbit eRF (Beaudet and Caskey 1971; Caskey et al. 1974). The 50 kDa protein band extracted from the gels and subjected to 2D gel electrophoresis was used for microsequencing. Four rabbit peptides generated after trypsinolysis were sequenced, proving the homogeneity of the purified rabbit eRF preparation (Frolova et al. 1994).

eRF1 protein family

Alignment of these four sequenced tryptic peptides of rabbit eRF with amino acid sequences from databanks is presented in Fig. 1. It is obvious from this comparison that peptides isolated from rabbit eRF1 are identical with, or very similar to, several proteins from various organisms. It was speculated based on genetic data that one of the proteins, yeast Sup45, belonging to this family might be involved in maintenance of translation fidelity (reviewed in Stansfield and Tuite 1994*a*) or (and) in translation termination (Inge-Vechtomov and Andrianova 1970), but the function of other proteins of this family were unknown. Therefore, to prove that this protein family is in fact eRFs, one should demonstrate RF activity of at least some of the members of this family.

eRF1 proteins from *Homo sapiens* (TB3-1, Grenett et al. 1992) and from *Xenopus laevis* (C11, Tassan et al. 1993) were expressed in yeast and *Escherichia coli*, respectively, and then purified. The purified human and frog eRF1 do possess RF activity in the in vitro RF assay (Tate and Caskey 1990) in the presence of any of the stop codons, while in the presence of a sense codon for Trp, UGG, structurally related to the stop codons UGA and UAG, they are inactive.

In eukaryotes, GTP is involved in translation termination (Beaudet and Caskey 1971; Konecki et al. 1977). However, the RF activity of *Xenopus* eRF1 (C11 protein) is GTP independent (Zhouravleva et al. 1995) and the proteins belonging to eRF1 family (Fig. 1) do not possess GTP-binding motifs (Frolova et al. 1994).

In prokaryotes, besides RF1 and RF2 recognizing the UAA/UAG and UAA/UGA stop codons, respectively (reviewed in Caskey 1980; Craigen et al. 1990), a third factor, termed RF3, has been identified in *E. coli* that stimulates the termination reaction and binds guanine nucleotides but is not codon specific (Milman et al. 1969). The *E. coli* gene encoding RF3 has been sequenced and it has been shown that this protein exhibits GTP-binding motifs (Grentzmann et al. 1994; Mikuni et al. 1994).

From the above-mentioned data, one may assume that eRF1 is not a single RF in eukaryotes and that another factor should exist that confers GTP requirement for termination reaction.

eRF3 protein family

Certain biological properties (tight association with ribosomes, low abundance towards ribosomal proteins, involvement in omnipotent suppression) of yeast Sup45 protein (Sup45p) are similar to Sup35 protein (Sup35p) (reviewed by Surguchov 1988). This may indicate that Sup35p could be a candidate for the putative nonidentified eRF since yeast Sup45p should be eRF1 (Frolova et al. 1994).

The *Saccharomyces cerevisiae SUP35* gene has been identified and sequenced (Kushnirov et al. 1988). Yeast Sup35p is composed of at least two domains (Kushnirov et al. 1988; Kikuchi et al. 1988; Wilson et al. 1988; Ter-Avanesyan et al. 1993): the amino (N) and the carboxy (C) terminal domains

Fig. 1. Alignment of the predicted amino acid sequences of the human Cl1 protein (HuCl1, Frolova et al. 1994), human TB3-1 protein (HuTB3-1) deduced from the TB3-1 clone (Grenett et al. 1992) and resequenced (Frolova et al. 1994), *X. laevis* Cl1 protein (XlCl1, Tassan et al. 1993), yeast Sup45 (Sup1) protein (YSUP45, Breining and Piepersberg 1986), and *Arabidopsis thaliana* Sup45-like protein (ArabSUP45; GenBank accession number X69375). Asterisks denote residues identical with the human TB3-1/Cl1 protein. Four peptides, *a*, *b*, *c*, and *d*, sequenced from rabbit 50 kDa polypeptide (rab.eRF) are scattered along the polypeptide chains of the protein family, indicating that the homology between rabbit eRF and the other proteins encompasses the entire length rather than a limited part of the polypeptide.

```
MADDPSAADRNVEIWKIKKLIKSLEAARGNGTSMISLIIPPKDQISRVAK  50 HuTB3-1/Cl1
**************************************************  50 XlCl1
*DNE---VEK*I****V***VQ***K**********V****GL*PLYQ*  47 YSUP1
***QE*--*K*I**********G**T**********M**R**VA**T*  48 ArabSUP1

                  a___________
MLADEFGTASNIKSRVNRLSVLGAITSVQQRLKLYNKVPPNGLVVYCGTI 100 HuTB3-1/Cl1
************************************************** 100 XlCl1
**T**Y***************S****T***************L***D*  97 YSUP1
******************Q***S****A***************L*T***  98 ArabSUP1

VTEEGKEKKVNIDFEPFKPINTSLILCDNKFHTEALTALLSDDSKFGFIV 150 HuTB3-1/Cl1
**********************Y********************** 150 XlCl1
I**D******TF*I**Y*******Y*********V*SE**QA*D****** 147 YSUP1
**DD******T**********A**Y*********P*NE**ES*D****** 148 ArabSUP1

                    b_____
IDGSGALFGTLQGNTREVLHKFTVDLPKKHGRGGQSALRFARLRMEKRHN 200 HuTB3-1/Cl1
************************************************** 200 XlCl1
M**Q*Q*T***SVS****T**********************E***** 197 YSUP1
M**N*T*****S********************************** 198 ArabSUP1

YVRKVAETAVQLFIS--GDKVNVAGLVLAGSADFKTELSQSDMFDQRLQS 248 HuTTB3-1/Cl1
**************--********************************** 248 XlCl1
*******V***N**T--N*****K**I*********D*AK*EL**P**AC 245 YSUP1
****T**L*T*FY*NPATSQP**S**I*************EL**P***A* 248 ArabSUP1

KVLKLVDISYGGENGFNQAIELSTEVLSNVKFIQEKKLIGRYFDEISQDT 298 HuTB3-1/Cl1
************************************************** 298 XlCl1
**ISI**V***************A*A*A***YV******EA********* 295 YSUP1
*I*NV**V***************A*I********************** 298 ArabSUP1

                                  c_________ d_____
GKYCFGVEDTLKALEMGAVEILIVYENLDIMRYVLHCQGTEEEKILYLTP 348 HuTB3-1/Cl1
**********************************R*N*S****T***** 348 XlCl1
**F*Y*ID******DL****K***F***E*I**TFKD--A*DNEVIKFAE 343 YSUP1
***V****************T***W*****N**E*KNNT*G*IV*KH*GK 348 ArabSUP1

____
EQEKDKSHFTDKETGQEHELIESMPLLEWFANNYKKFGATLEIVTDKSQE 398 HuTB3-1/Cl1
**********************************S**************** 398 XlCl1
PEA****FAI**A****MDVVSEE*****I*AN**N******FI****S* 393 YSUP1
D**NNQ*N*H*A**NA*L*VQ*K*********E**RF*C***F**N**** 398 ArabSUP1

GSQFVKGFGGIGGILRYRVDFQGMEYQGGDDEFFDLDDY            437 HuTB3-1/Cl1
**********************D***V********** 437 XL1Cl1
GA***T******AM***K*N*EQL-VDESE**YY*E*EGSDYDFI      437 YSUP1
****CR********L***QL*MRTFD-ELS*G*VYE-*SD           435 ArabSUP1
```

(253 and 432 amino acids, respectively) and the C-domain exhibits GTP-binding motifs (Kushnirov et al. 1988; Kikuchi et al. 1988; Wilson et al. 1988, Samsonova et al. 1991). The C-domain of Sup35-like proteins is conserved even between highly remoted species like yeast and human (Hoshino et al. 1989), contrary to the N-terminal parts of these proteins that are entirely different. The functional dissimilarity of the N- and C-domains of yeast Sup35p has been discussed thoroughly (Doel et al. 1994; Ter-Avanesyan et al. 1994).

The yeast genes, *SUP45* (SUP1) and *SUP35* (SUP2), being affected by mutations acquire omnipotent suppressor activity (Inge-Vechtomov and Andrianova 1970; Surguchov et al. 1984), and it was supposed based on genetic data that yeast Sup45p and Sup35p might both be involved in the maintenance of translational fidelity (reviewed in Stansfield and Tuite 1994*a*). The old idea (Inge-Vechtomov and Andrianova 1970) concerning participation of these proteins in translation termination could now be reanimated in view of current demonstration of the eRF1 activity for protein family including yeast Sup45p and the lack of GTP-binding motifs in eRF1 (Frolova et al. 1994).

Using yeast genomic *SUP35* DNA (Kushnirov et al. 1988) and *GSPT1* cDNA, a human analog of yeast *SUP35* (Hoshino et al. 1989), as probes, a *X. laevis* cDNA library has been screened and one of the positive clones that contained the longest insert (~2.3 kb) has been sequenced (Zhouravleva et al. 1995). The deduced amino acid sequence of the C-domain of the *X. laevis* Sup35p-like protein (designated as Sup35Cp) is similar to that of human GSPT1p (Hoshino et al. 1989), *S. cerevisiae* (Kushnirov et al. 1988), and *Pichia pinus* (Kushnirov et al. 1990) Sup35p (Fig. 2), indicating the high conservation of the primary structure of this domain. All of these proteins exhibit GTP-binding motifs (G1–G4) in the C-domain similar to those in the other G proteins, in particular in elongation factor EF-1α (Kushnirov et al. 1988; Samsonova et al. 1991) and prokaryotic RF3 (Grentzmann et al. 1994; Mikuni et al. 1994), that have to be responsible for guanine nucleotide binding and GTP hydrolysis (reviewed in Bourne et al. 1991).

For further experiments, a construct containing the cDNA encoding the *X. laevis* Sup35Cp starting from Met116 (Fig. 2) has been generated. The *E. coli* expressed and affinity-purified *X. laevis* Sup35Cp yields one predominant band (~58 kDa) on SDS–PAGE after Coomassie blue staining. The purified Sup35Cp tested in the in vitro RF assay (Table 1) possesses no RF activity but it considerably enhances the activity of *X. laevis* eRF1 (C11 protein) at a nonsaturating level of all three stop codons. This stimulation is entirely GTP dependent (Table 2) and is inhibited by a nonhydrolyzable analog of GTP, GTPγS, while GDP, GMP, or ATP have no effect. Rabbit polyclonal antibodies raised against *X. laevis* Sup35Cp significantly inhibit RF-stimulating activity of Sup35Cp and do not diminish the activity of eRF1 (C11). Consequently, besides conferring GTP dependence, the second protein, eRF3, allows the concentration of the stop codons to be reduced considerably in the in vitro RF assay governed by eRF1 (Zhouravleva et al. 1995).

Two features of *X. laevis* Sup35Cp/eRF3 (GTP-binding motifs and stimulation of RF activity at limiting concentrations of stop codons) resemble those of *E. coli* RF3 (Grentzmann et al. 1994; Mikuni et al. 1994). For these reasons, we term the *X. laevis* GTP-binding protein and other proteins of this family (Fig. 2) as eRF3. However, the properties of eRF3 and prokaryotic RF3 exhibit certain distinct features: GTP enhances the eRF3 activity but inhibits the prokaryotic RF3 (Mikuni et al. 1994) and part of the yeast *SUP35*-like gene encoding Sup35Cp is essential for cell viability (Kushnirov et al. 1988), although *E. coli* gene-encoding RF3 is nonessential (Grentzmann et al. 1994; Mikuni et al. 1994). Obviously, since yeast Sup35p belongs to the same structural family (Fig. 2), it should also be termed as eRF3/Sup35p; however, its RF activity has not yet been demonstrated in the appropriate assay system. Based on the previous results (Frolova et al. 1994), the same suggestion concerning Sup35p function has been made by Stansfield and Tuite (1994*b*) but has not been proved biochemically.

Here, we do not consider the role of the N-domain in the function of Sup35p. It is known that this part of yeast Sup35p might be functionally antagonistic towards its own C-domain (Doel et al. 1994; Ter-Avanesysan et al. 1994). Moreover, the properties of the full-length Sup35p of higher eukaryotes might differ from the functional activity of the C-domain; these aspects of the function of Sup35p in the higher eukaryotes would be considered elsewhere.

Concluding remarks

We conclude that in vertebrates (and most likely in eukaryotes in general), two factors (eRF1 and eRF3) govern the translation termination process as in prokaryotes (RF1/RF2 and RF3). The first factor, eRF1, recognizes directly or indirectly all three stop codons in mRNA and catalyzes the peptidyl-tRNA hydrolysis while this reaction proceeds more efficiently and becomes GTP dependent due to participation of the second factor, eRF3, in the overall reaction. When two proteins are involved in one and the same reaction, one may anticipate their interaction. In fact, we observed that *X. laevis* eRF1 and eRF3 bind to each other (Zhouravleva et al. 1995). This observation provokes the assumption that eRF1 and eRF3 are also able to form a complex in vivo. To be more precise, we assume that the C-domain of the eRF3 is involved in complex formation with eRF1.

How does the eRF1/eRF3 associate function in termination? We propose that a quaternary complex composed of the ribosome, eRF1, eRF3, and GTP is a prerequisite for the ribosome competence in translation termination. In turn, this eRF1–eRF3–GTP complex exhibits higher affinity towards the stop codons in mRNA, blocking possible binding of tRNAs to nonsense codons. From this model, it follows that elevated levels of eRF1 and eRF3 in cells should cause antisuppressor activity.

We have demonstrated that eRF1 family is structurally highly conserved (Frolova et al. 1994). Since eRF1 binds to the C-domain of eRF3, one may anticipate the high conservation of eRF3 structure too, at least at the C-domain. This prediction is in agreement with sequence data (Fig. 2) and genetic complementation data (Zhouravleva et al. 1995).

The structural nonresemblance of prokaryotic and eukaryotic RFs coupled with their functional similarity raises an intriguing question about the origin of the translation termination machinery in eukaryotes. One of the ideas proposed earlier (Frolova et al. 1994) concerns the origin of prokaryotic

Fig. 2. Alignment of the predicted amino acid sequences of human GSPT-1p (HsGSPT1, Hoshino et al. 1989), *X. laevis* (Xl*SUP35*, Zhouravleva et al. 1995), *S. cerevisiae* (Sc*SUP35*, Kushnirov et al. 1988), and *Pichia pinus* (Pp*SUP35*, Kushnirov et al. 1990) Sup35Cp. Conserved (G1–G4) G-domain motifs (Bourne et al. 1991) are indicated by solid lines. Conserved amino acids are boxed. Met1 of *X. Laevis* Sup35Cp corresponds to Met116 deduced from the sequenced *X. laevis* Sup35 cDNA clone (Zhouravleva et al. 1995).

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HsGSPT1                MELSEPI--VENGETEMSPEESWEHKEEISEAEP--GGGSLGDGRPP    43
XlSUP35                MDVSEPV--VENGETEMSPEESWDHKEEPIEAEP--GGGSEGDGGTT    43
ScSUP35  EPTKVEEPVKKEE-----KPV-QTE-------EKTEEKS--ELPKVED-LKISESTHNTNNAN  231
PpSUP35  AENKVEEEESKVEAPTAAKPVSESEPPASTPKTEAKASKEVAAAAAALK-KEVSQAKKESN  289
                                    G1

HsGSPT1  EESAHEMMEEEEEEIPKPKSVVAPPGAPKKEHVNVVFIGHVDAGKSTIGGQIMYLTGMVDK  103
XlSUP35  EEGTSEMMEEEEEEIPKPKTIVVPPDAPKKEHVNVVFIGHVDAGKSTIGGQIMYLTGMVEK  103
ScSUP35  --VTSADALIKEQEEEVDDEVVNDMFGGKDHVSLIFMGHVDAGKSTMGGNLLYLTGSVDK  289
PpSUP35  --VTNADALVKEQEEQIDASIVNDMFGGKDHMSIIFMGHVDAGKSTMGGNLLFLTGAVDK  347
                                  G2                  G3

HsGSPT1  RTLEKYEREAKEKNRETWYLSWALDTNQEERDKGKTVEVGRAYFETEKKHFTILDAPGHK  163
XlSUP35  RTLEKYEREAKEKNRETWYLSWALDTNQEERDKGKTVEVGRAYFETEKKHFTILDAPGHK  163
ScSUP35  RTIEKYEREAKDAGRQGWYLSWVMDTNKEERNDGKTIEVGKAYFETEKRRYTILDAPGHK  349
PpSUP35  RTVEKYEREAKDAGRQGWYLSWIMDTNKEERNDGKTIEVGKSYFETDKRRYTILDAPGHK  407
                                                    G4

HsGSPT1  SFVPNMIGGASQADLAVLVISARKGEFETGFEKGGQTREHAMLAKTAGVKHLIVLINKMD  223
XlSUP35  SFVPNMIGGASQADLAVLVISARKGEFETGFEKGGQTREHAMLAKTAGVKHLIVLINKMD  223
ScSUP35  MYVSEMIGGASQADVGVLVISARKGEYETGFERGGQTREHALLAKTQGVNKMVVVVNKMD  409
PpSUP35  LYISEMIGGASQADVGVLVISSRKGEYEAGFERGGQSREHAILAKTQGVNKLVVVINKMD  467

HsGSPT1  DPTVNWSNERYEECKEKLVPFLKKVGFNPKKDIHFMPCSGLTGANLKEQSDF--CPWYIG  281
XlSUP35  DPTVNWSNDRYEECKEKLVPFLKKVGFNPKKDIYFMPCSGLTGANLKEPVET--CPWYIG  281
ScSUP35  DPTVNWSKERYDQCVSNVSNFLRAIGYNIKTDVVFMPVSGYSGANLKDHVDPKECPWYTG  469
PpSUP35  DPTVNWSKERYEECTTKLAMYLKGVGYQ-KGDVLFMPVSGYTGAGLKERVSQKDAPWYNG  526

HsGSPT1  LPFIPYLDNLPNFNRSVDGPIRLPIVDKYKDMGTVVLGKLESGSICKGQQLVMMPNKHNV  341
XlSUP35  LAFISYLDSLPNFNRSLDGPVRLPIVDKYKDMGTVILGKLESGSICKGQQLAMMPNKHIV  341
ScSUP35  PTLLEYLDTMNHVDRHINAPFMLPIAAKMKDLGTIVEGKIESGHIKKGQSTLLMPNKTAV  529
PpSUP35  PSLLEYLDSMPLAVRKINDPFMLPISSKMKDLGTVIEGKIESGHVKKGQNLLVMPNKTQV  586

HsGSPT1  EVLGILSD-DVETDTVAPGENLKIRLKGIEEEEILPGFILCDPNNLCHSGRTFDAQIVII  400
XlSUP35  EVLSLLSD-EVETELVAPGENLK-RLKGIEEEEILPGFILCDPNNLCHSGRTFDAQIVII  400
ScSUP35  EIQNIYNETENEVDMAMCGEQVKLRIKGVEEEDISPGFVLTSPKNPIKSVTKFVAQIAIV  589
PpSUP35  EVTTIYNETEAEADSAFCGEQVRLRLRGIEEEDLSAGYVLSSINHPVKTVTRFEAQIAIV  646

HsGSPT1  EHKSIICPGYNAVLHIHTCIEEVEITALICLVDKKSGEKSKTRPRFVKQDQVCIARLRTA  460
XlSUP35  EHKSIICPGYNAVLHIHTCIEEVEITALICMVDKKSGEKSKTRPRFVKQDQVCIARLETA  460
ScSUP35  ELKSIIAAGFSCVMHVHTAIEEVHIVKLLHKLEKGTNRKSKKPPAFAKKGMKVIAVLETE  649
PpSUP35  ELKSILSTGFSCVMHVHTAIEEVTFTQLLHNLQKGTNRRSKKAPAFAKQGMKIIAVLETT  706

HsGSPT1  GTICLETFKDFPQMGRFTLRDEGKTIAIGKVLKLVPEKD                        499
XlSUP35  GTICLETFKDFPQMGRFTLRDEGKTIAIGKVLKLVPEKD                        499
ScSUP35  APVCVETYQDYPQLGRFTLRDQGTTIAIGKIVKIA-E                          685
PpSUP35  EPVCIESYDDYPQLGRFTLRDQGQTIAIGKVTKLL                            741
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Table 1. Effects of *X. laevis* Sup35Cp on the in vitro RF activity* of *X. laevis* Cl1 (eRF1) protein.

Protein(s)	Sup35Cp added (μg)	Stop codon added (μM) UAAA 50	UAAA 5	UAGA 50	UAGA 5	UGAA 50	UGAA 5
Cl1	—	0.44	0.01	0.71	0.02	0.48	0.01
— Sup35Cp	0.3	0	0	0	0	0	0
Cl1 + Sup35Cp	0.1	—	0.38	—	0.50	—	0.37
Cl1 + Sup35Cp	0.2	—	0.52	—	0.85	—	0.51
Cl1 + Sup35Cp	0.3	—	0.56	—	0.88	—	0.63

Note: The part of *X. laevis SUP35* cDNA corresponding to the C-domain starting from Met116 of the sequenced *X. laevis* cDNA clone (Zhouravleva et al. 1995) that corresponds to Met1 indicated on Fig. 2 was recloned in *NheI/XhoI* sites of pET21b plasmid (Novagen). A His-tagged tail was present at the C-terminus of the protein. The expression and purification of the *X. laevis* Sup35Cp were performed as described for *X. laevis* Cl1 (eRF1) protein (Frolova et al. 1994). RF-stimulating activity was measured in vitro as a stop-codon-dependent hydrolysis of f[^{35}S]Met-tRNA^{fmet} associated with AUG–80S–ribosome complex (Caskey et al. 1974; Tate and Caskey 1990). The incubation mixture (25 μL) contained 20 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 8 mM NH_4Cl, 0.1 mM GTP, 1.5 pmol of f[^{35}S]Met-tRNA^{fmet}–AUG–ribosome complex, 0.2 μg of *X. laevis* Cl1 protein (eRF1), 5 μM of stop codon (that corresponded to about 10% of the saturation level needed for the complete fMet release with Cl1 but without Sup35p), and indicated amounts of the purified *X. laevis* Sup35Cp. The expression and purification of *X. laevis* Sup35C protein were performed as described (Zhouravleva et al. 1995). The RF activity of Cl1 was measured in the same incubation mixture except the concentration of the stop codon that was 50 μM (saturation level). The RF activity was calculated as the amount of f[^{35}S]Met released in the presence of stop codon; the pmol of f[^{35}S]Met released in the absence of stop codon has been subtracted from all values. Taken alone, Sup35Cp was completely inactive at varying concentrations and in the presence of any of the three stop codons in the in vitro RF assay.

*f[^{35}S]Met released, pmol.

Table 2. Influence of GTP and GTPγS on the RF activity* of Cl1 (eRF) protein and the RF-stimulating activity of Sup35Cp.

Protein(s)	Guanine nucleotides added (0.1 mM)	Stop codon added (μm) UAAA	UGAA	UAGA
Cl1	None	1.00	0.87	0.90
Cl1	GTP	0.86	1.01	0.75
Cl1	GTPγS	0.82	0.93	0.72
Cl1 + Sup35Cp	None	0.02	0.05	0.01
Cl1 + Sup35Cp	GTP	0.97	0.93	0.88
Cl1 + Sup35Cp	GTPγS	0.05	0.06	0.03

Note: The RF activity of *X. laevis* Cl1 (eRF1) protein and stimulating activity of *X. laevis* Sup35Cp was measured as described in Table 1.

*f[^{35}S]Met released, pmol.

and eukaryotic translation termination systems as being independent processes in evolution.

The observation that eRF3 by itself has no RF activity but binds to eRF1 points to the possibility that eRF3 does not recognize stop codons and is involved in termination indirectly, via interaction with eRF1. Since eRF3 is totally inactive in the absence of GTP, we assume that binding and hydrolysis of GTP controls the interaction of the eRFs.

It remains to be studied whether GTP is split at once with peptidyl-tRNA hydrolysis to release free nascent polypeptide and free tRNA or whether its hydrolysis is used to induce dissociation of the ribosomal subunits as proposed in many previous works.

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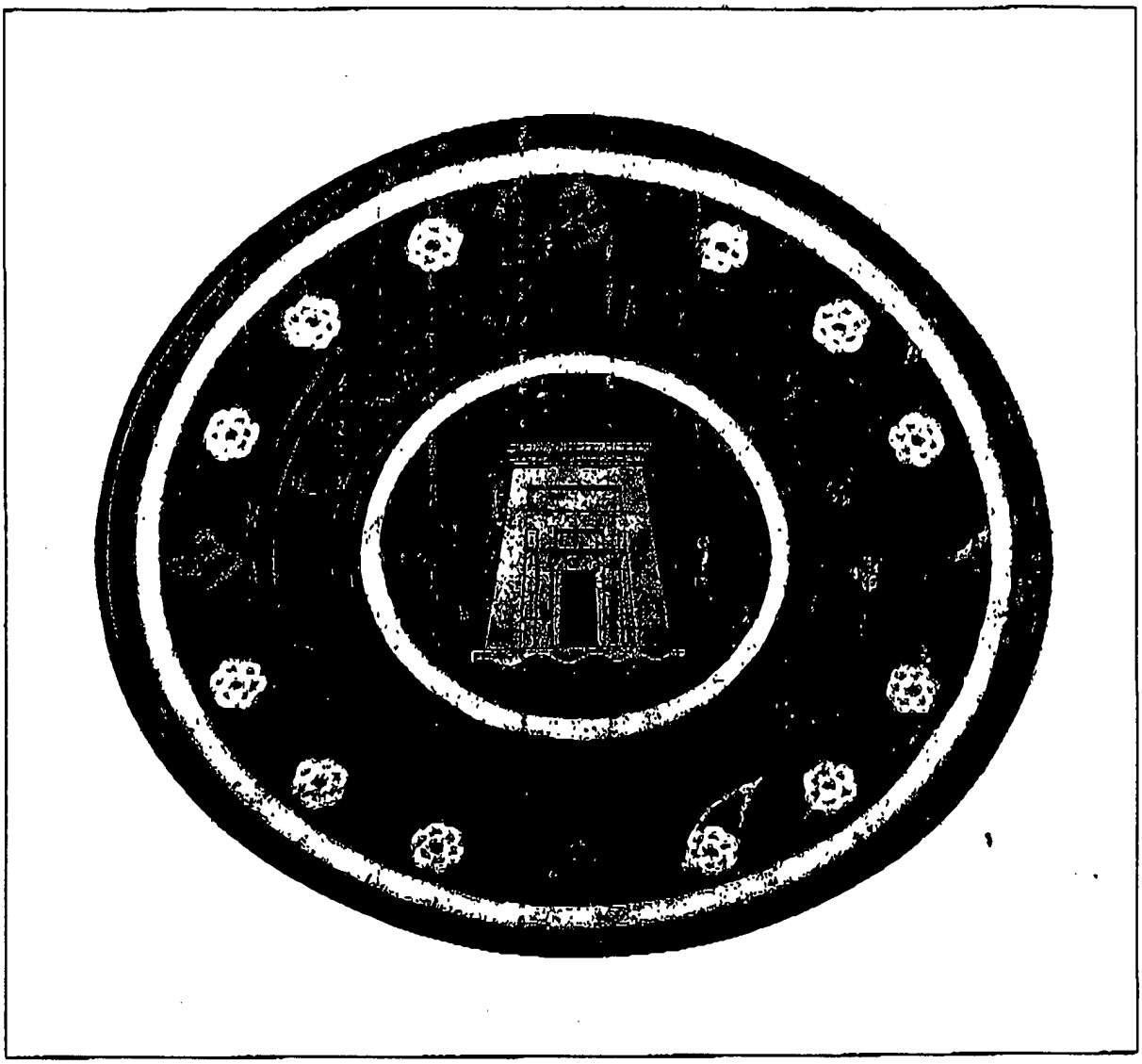

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Genetics

Mammalian orthologues of a yeast regulator of nonsense transcript stability

(RNA degradation/RNA metabolism/nonsense mutation/premature termination codon)

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ABSTRACT **All eukaryotes that have been studied to date possess the ability to detect and degrade transcripts that contain a premature signal for the termination of translation. This process of nonsense-mediated RNA decay has been most comprehensively studied in the yeast *Saccharomyces cerevisiae* where at least three trans-acting factors (Upf1p through Upf3p) are required. We have cloned cDNAs encoding human and murine homologues of Upf1p, termed rent1 (regulator of nonsense transcripts). Rent1 is the first identified mammalian protein that contains all of the putative functional elements in Upf1p including zinc finger-like and NTPase domains, as well as all motifs common to members of helicase superfamily I. Moreover, expression of a chimeric protein, containing the central region of rent1 flanked by the extreme N and C termini of Upf1p, complements the Upf1p-deficient phenotype in yeast. Thus, despite apparent differences between yeast and mammalian nonsense-mediated RNA decay, these data suggest that the two pathways use functionally related machinery.**

Many observations have suggested that the low abundance of nonsense transcripts in yeast is due to cytoplasmic mRNA degradation, intimately involving translation by the ribosome machinery (1). At least three trans-acting factors (Upf1p through Upf3p) are required and loss of function of any one restores the stability of nonsense transcripts in a manner indistinguishable from double disruptions, suggesting that the Upf proteins perform complementary critical functions in a common pathway (2, 3). Apparent differences exist between nonsense-mediated RNA decay (NMRD) in yeast and the highly analogous activity that is observed in mammals (for review, see ref. 4). Notably, for many mammalian genes and mutations, the reduced abundance of cytoplasmic nonsense transcripts can be fully accounted for by a reduced abundance in the nucleus despite normal rates of transcription. Other lines of evidence for a nuclear localization for nonsense transcript recognition and decay include a dependence of the process upon intron sequences, normal stability of nonsense transcripts once in the cytoplasm and polysome bound, and the ability of nonsense codons to inhibit pre-mRNA splicing or alter exon definition by the splicing machinery (4–8). Yet, evidence exists that inhibition of translation initiation or elongation or read-through of premature termination codons by the introduction of suppressor tRNAs can abrogate NMRD, at least in part, in mammalian cells (for review, see ref. 4). A unifying theory that reconciles all of these data is lacking. Either the mechanism of NMRD in mammalian cells is extremely heterogeneous, perhaps even transcript-, cell type-, or genotype-specific, or the lack of critical information precludes full conceptualization of the process. To determine whether yeast and mammalian NMRD utilize similar machinery, we sought to identify a mammalian homologue of yeast Upf1p, the best characterized yeast component of the NMRD pathway that has demonstrated nucleotide binding, ATPase, and 5′ → 3′ helicase activity (9).

METHODS

Cloning of the Human RENT1 cDNA. Submission of Upf1p sequence to the XREF database genome cross-referencing effort (10) resulted in the identification of a human expressed sequence tag (GenBank accession no. F06433) that encodes a protein with significant similarity to Upf1p. Full-length RENT1 cDNA clones were isolated from an adult human heart cDNA library using the GENETRAPPER cDNA positive selection system (Life Technologies, Gaithersberg, MD). Screening was carried out according to the manufacturer's instructions with oligonucleotide 23–5-2a (5′-CTTTGACAGGATGCAGAGCGC-3′). Sequencing was performed using a Perkin–Elmer Applied Biosystems Division model 373a automated DNA sequencer by following manufacturer's protocols.

Partial Cloning of the Murine *Rent1* Gene. A large (≈15.5 kb) murine *Rent1* genomic clone was identified by probing a 129/SV strain mouse genomic library (Stratagene) with a radiolabeled human cDNA fragment. An ≈8.2-kb *Bam*HI restriction fragment was subcloned into pBluescriptII/SK+ (Stratagene) and sequenced as described above. A 7-kb *Bam*HI–*Not*I restriction fragment from the 3′ region of the same genomic clone was subcloned and approximately 4.4 kb was sequenced. Coding sequence was identified by aligning the conceptual translation of the human cDNA with that for the murine genomic clone using the MACVECTOR 4.5.3 package of sequence analysis software (Kodak).

Expression Pattern of RENT1. Prepared multiple human tissue Northern blots were obtained from CLONTECH and probed with a 300-bp RENT1 cDNA fragment (encoding aa 644–745) or a β-actin cDNA probe (CLONTECH) according to instructions supplied by the manufacturer.

Chromosomal Mapping. *Rent1* was mapped in mouse by the Genome Cross-Referencing Group as D8Xrf83 and relevant data may be viewed on the World Wide Web at URL http://www.ncbi.nlm.nih.gov/XREFdb/. The 1.6-kb cDNA insert from Genexpress clone c-18a11 containing a human expressed sequence tag (GenBank accession no. F06433) was hybridized to filters derived from the Jackson Laboratory BSS backcross DNA panel. The human probe detected a C57BL/6J-specific 1.6-kb *Taq*I restriction fragment that was used to

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Abbreviations: NMRD, nonsense-mediated RNA decay; UTR, untranslated region; PGA, Pro-Gly-Ala.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U65533 for RENT1 cDNA).

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determine the map position of the murine gene. The closest mapped murine gene was *Ncan* with zero crossovers in 83 N_2 progeny. The 1.6-kb expressed sequence tag insert also detected 10.5-, 4.0-, and 2.8-kb human chromosome 19-specific *Hin*dIII restriction fragments within the NIGMS human/rodent somatic cell hybrid panel (Version 2, Coriell Cell Respositories).

Complementation Studies in Yeast. To construct pMET25-RENT, an *Xho*I fragment containing the entire RENT1 coding region was inserted into the *Sma*I site of p426MET25 (11). The *Bam*HI fragment from pBM272UPF1 (a gift from M. Culbertson, University of Wisconsin, Madison, WI) was inserted into the *Bam*HI site of p426MET25 to create pMET25-UPF1. pMET25-CHIMERA was constructed using high-fidelity *Taq* polymerase (Pfu, Stratagene) with the addition of restriction sites for cloning as follows: The 5′ region [5′ untranslated region (UTR) and sequence encoding aa 1–59] of Upf1p was amplified by PCR with UPF-Bam-S (5′-GGATCCCATCAGGAAAGAAG-3′) and UPF-Sal-AS (5′-GTCGACTGAAGCTGAAGGCGAACGG-3′). The 3′ region of Upf1p (encoding aa 854–971 and 3′ UTR) was amplified by UPF-Not-S (5′-GCGGCCGCGAAAGACTGAACGGCCAAT-3′) and UPF-Hind-AS (5′-AAGCTTAT-CCAAAGTATATTGGACCGG-3′). The central region of rent1 (encoding aa 121–917) was amplified with RNT-SalS-1 (5′-ACGCGTCGACCACGCCTGCAGTACTGTGGAATAC-3′) and RNT-NotAS-1 (5′-ATAAGAATGCGGCCGCGGCTGCTGAACGCATGAGGCTCTCACG-3′). Each PCR product was subcloned into the *Sma*I site of pBluescriptII/SK+ (Stratagene), excised with the appropriate restriction enzymes, and ligated into p426MET25 digested with *Bam*HI and *Hin*dIII. pMET25-y5′ was constructed by inserting the 5′ UPF1 *Bam*HI–*Sal*I fragment into *Bam*HI/*Sal*I-digested p426MET25. Transformants were grown in liquid culture to a similar OD_{600} value and 4 μl of serially diluted cultures was plated on solid medium lacking methionine (for promoter induction), uracil (for selection of transformants), and histidine (as a marker for the stability of *his4-38* transcripts).

RESULTS

Upf1p sequence was submitted to the XREF database genome cross-referencing effort (10). In this manner we identified a human expressed sequence tag, derived from a normal human infant brain cDNA library, that encodes a protein with significant similarity to Upf1p. Subsequent screening of an adult human heart cDNA library resulted in the isolation of two clones with an insert size of ≈3.7 kb. Direct sequencing predicts an ORF of 3354 bp with 231 bp of 5′ UTR and 103 bp of 3′ UTR, excluding the poly(A) tail (Fig. 1). The putative initiating methionine is encoded by an ATG that occurs within the context of a Kozak consensus sequence (12), 23 codons

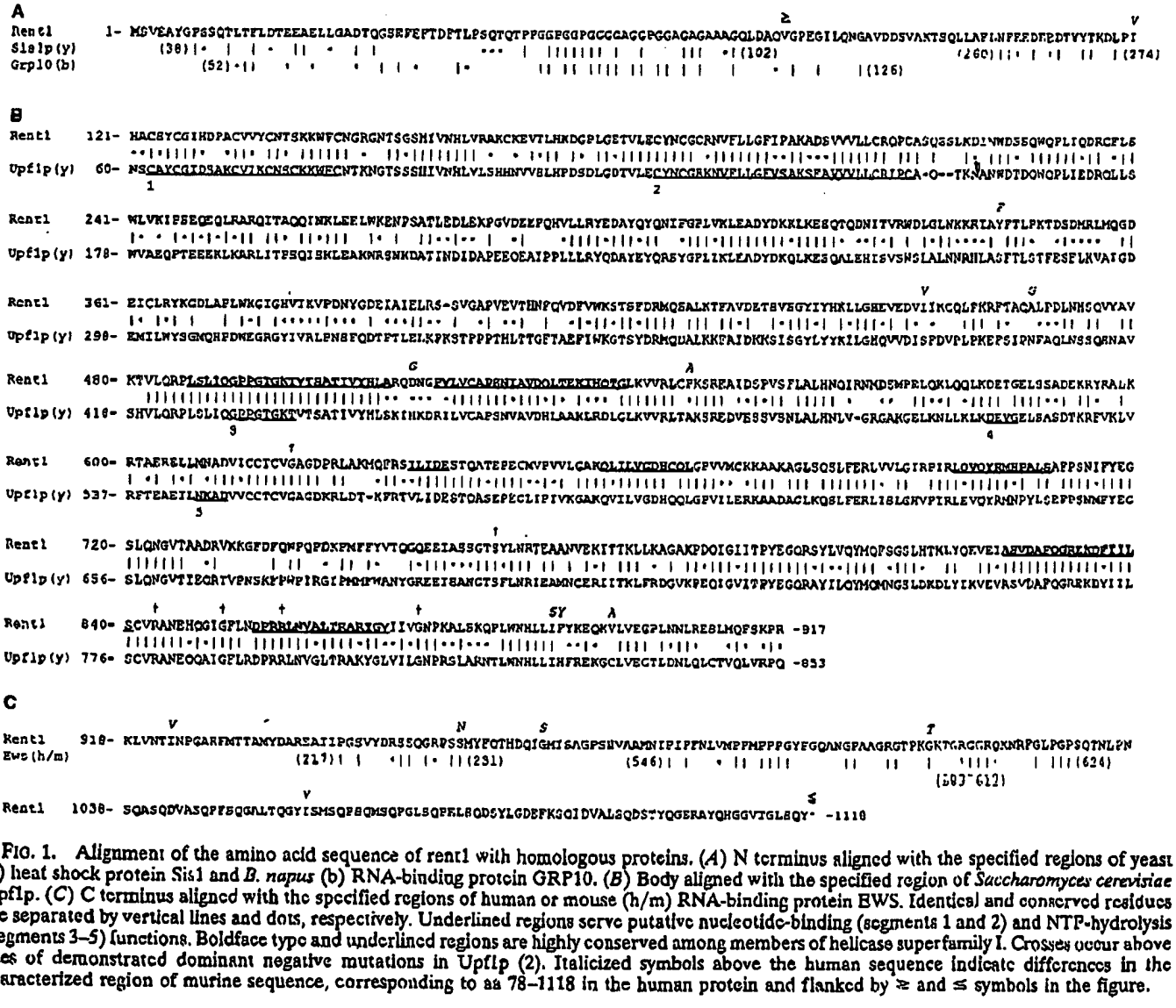

FIG. 1. Alignment of the amino acid sequence of rent1 with homologous proteins. (*A*) N terminus aligned with the specified regions of yeast (*y*) heat shock protein Sis1 and *B. napus* (b) RNA-binding protein GRP10. (*B*) Body aligned with the specified region of *Saccharomyces cerevisiae* Upf1p. (*C*) C terminus aligned with the specified regions of human or mouse (h/m) RNA-binding protein EWS. Identical and conserved residues are separated by vertical lines and dots, respectively. Underlined regions serve putative nucleotide-binding (segments 1 and 2) and NTP-hydrolysis (segments 3–5) functions. Boldface type and underlined regions are highly conserved among members of helicase superfamily I. Crosses occur above sites of demonstrated dominant negative mutations in Upf1p (2). Italicized symbols above the human sequence indicate differences in the characterized region of murine sequence, corresponding to aa 78–1118 in the human protein and flanked by ≥ and ≤ symbols in the figure.

downstream of an in-frame translational terminator. Three tandem consensus polyadenylylation signals (AATAAA) begin at positions 63, 70, and 76 of the 3′ UTR, with initiation of the poly(A) tail at position 104. Northern blot analysis demonstrated that the transcript is expressed in all human adult tissues that were tested (Fig. 2), as expected for a putative critical component of the apparently ubiquitous NMRD pathway.

We have assigned the names RENT1 and rent1 (regulator of nonsense transcripts) to the human gene and protein, respectively. Although divergence is seen at the extreme N and C termini, the large central regions of Upf1p and rent1 (residues 60–853 and 121–917, respectively) show 58% identity and 80% conservation (Fig. 1). Moreover, to our knowledge, rent1 is the first identified mammalian protein that contains all of the putative functional elements found in Upf1p including the cysteine-rich zinc finger-like domains that may participate in nucleotide binding, the domains with putative NTPase activity, and the motifs common to members of helicase superfamily I (9). All residues shown to have dominant negative activity when mutated in Upf1p (3) are identical in rent1 (Fig. 1).

The N terminus of rent1 contains a region composed entirely of proline, glycine, and alanine (PGA) residues not found in Upf1p (Fig. 1). While the function of this region is unknown, PGA-rich stretches have been found to act as direct transcriptional repressors (13). Alternatively, the helix-disturbing properties imposed by the high PG content may confer a favorable conformation to the molecule. If one excludes the PGA-rich region, certain similarities are evident between the N termini of rent1 and Upf1p. Both are relatively rich in serines and threonines (19 versus 24%, respectively) and acidic residues (aspartic acid or glutamic acid; 22 versus 18%, respectively), features commonly seen in nucleotide-binding proteins with transactivation or transcriptional regulation properties (14, 15). Both C termini are rich in serines and glutamines (21 versus 18%, respectively) but the occurrence of the majority of these residues as SQ dipeptides ($n = 14$) is unique to rent1. Many RNA recognition motif (RRM)-containing proteins have glutamine-rich regions that are postulated to regulate multiple aspects of RNA processing (16). BLAST analysis (17) of the divergent N- and C-terminal sequences of rent1 reveals homology to multiple proteins that interact with RNA and regulate its processing. These include the Sis1 heat shock protein of yeast ($P = 5.6e-07$), the Gr10 RNA-binding protein of *Brassica napus* ($P = 1.2e-04$), and the human or mouse EWS RNA-binding protein ($P = 8.9e-03$) (18–20) (Fig. 1).

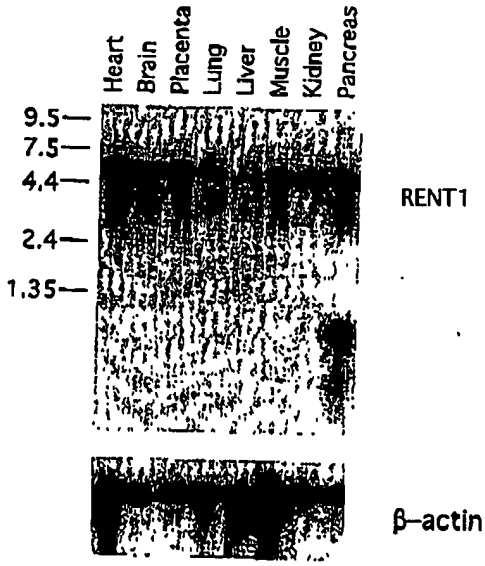

FIG. 2. Expression of RENT1. Northern blot analysis of poly(A) RNA shows a predominant transcript of approximately 5.4 kb in all of the indicated adult tissues. A less intense signal is seen at approximately 3.7 kb in all lanes, with an additional and significantly smaller hybridizing transcript unique to the pancreas. Additional tissues tested (spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte) all showed predominant ≈5.4-kb and less intense 3.7-kb bands, except in testes where equally intense signals were observed (data not shown).

A large (≈15.5 kb) murine *Rent1* genomic clone was identified upon screening of a 129/SV strain mouse genomic library (Stratagene). Conceptual translation and homology analysis of the 12.6 kb that has been sequenced shows that 1041 residues of the predicted murine protein, corresponding to residues 78–1118 in the human sequence, are encoded by 22 exons. Remarkably, only 14 of 1041 residues differ between the characterized portions of the human and murine proteins (Fig. 1). Alignment of the seven regions common to members of helicase superfamily I (21) for Upf1p, its homologues in human, mouse, and *Schizosaccharomyces pombe* (22, 23), along with Sen1 and Mov-10 (24, 25), reveals a strikingly conserved consensus that allows definition of this group of proteins as a distinct subset within the superfamily (Fig. 3).

To explore the functional properties of rent1, we determined whether its expression in yeast deficient for Upf1p activity could restore NMRD using a modification of the allosuppression assay originally used to identify Upf1p (2). We utilized the PLY38 strain (*MATa ura3-52 his4-38 SUF1-1 upf1-2*) that harbors a +1 frameshift mutation in the *HIS4* transcript and a tRNA frameshift suppressor with decreased efficiency at elevated temperatures (2). This strain can grow in histidine-deficient medium due to the combination of tRNA suppressor activity and the stability of *his4-38* mRNA in the absence of Upf1p. Reconstitution with Upf1p activity decreases *his4-38* message abundance and hence causes growth failure at high culture temperatures that are less permissive for suppressor tRNA function.

Transformation of yeast strain PLY38 with a rent1 expression construct failed to complement the yeast Upf1p-deficient phenotype (Fig. 4). The same result was obtained when the yeast ADH1 UTRs were placed flanking the entire rent1 coding sequence (data not shown). To test whether the divergence at the extreme N and C termini conferred species-specific functional constraints, we prepared a construct (pMET-Chimera) encoding the Upf1p 5′ UTR and N terminus (aa 1–59), the body of the human protein (aa 121–917), and the Upf1p C terminus (aa 854–971) and 3′ UTR. A dramatic inhibition of growth was seen in pMET25-Chimera transformants at elevated temperatures (Fig. 4).

DISCUSSION

We have identified strong human and murine homologues of Upf1p that contain all of the putative functional domains in the yeast protein including those believed to confer nucleotide binding, NTPase, and helicase activities. While purified Upf1p has been shown to have each of these properties, their precise role in the recognition and/or degradation of nonsense transcripts remains unknown. The ability of a chimeric protein, composed largely of the homologous domains in rent1, to complement the Upf1p-deficient phenotype in yeast provides significant evidence that rent1 is a mammalian orthologue of yeast Upf1p. It remains to be determined whether structural and functional divergence between Upf1p and rent1 accounts for the observed differences between yeast and mammalian NMRD. While Upf1p localizes predominantly to the cytoplasm (26), the subcellular localization of rent1 has not yet been determined. Of note is the fact that rent1 contains a peptide sequence, KKLK(X17)KKR, that is similar to the

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                          I(A)                                  Ia                    II(B)
CONSENSUS I   --UU-A-AGTGKT--U---U--U-        -U-----T--AU--U---U---U        UUUDE
                    G P S  S

Sen1          FSLIQGPPGTGKTKTILGIIGYFL  24  KILICAPSNAAVDEICLRLKSGV  159  VIIDE  18
Mov-10        PYIIFGPPGTGKTVTLVEAIKQVV   2  HILACAPSNSGADLLCQRLRVHL   70  IFIDE  21
Upf1p(S.c)    LSLIQGPPGTGKTVTSATIVYHLS   5  RILVCAPSNVAVDHLAAKLRDLG   92  VLIDE  18
     (S.p)    LSLIQGPPGTGKTVTSASVVYHLA  11  PVLVCAPSNVAVDQLAEKIHRTG   94  VLIDE  18
Rent1(human)  LSLIQGPPGTGKTVTSATIVYHLA   5  PVLVCAPSNIAVDQLTEKIHQTG   94  VLIDE  18
Rent1(mouse)  LSLIQGPPGTGKTVTSATIVYHLA   5  PVLVCAPSNIAVDQLTEKIHQTG   94  VLIDE  18
CONSENSUS II  LSLIQGPPGTGKTVTSAtIVYHLa        pvLVCAPSN-AVDqL-eKlh-tG        VLIDE
                                               i                 i

                          III                   IV                    V                    VI
CONSENSUS I   -U---GD--Q-          L---YR------        -TU---QG-------U-        -----YVAUTR-----
                                    V   F                S   K                    P G S
Sen1          RCIMVGDPNQL  30  LDVQYRMHPSIS  115  NTIDGFQGQEKEIILIS  15  DFRRMNVALTRAKTSI
Mov-10        QLVLAGDPRQL  43  LLRNYRSHPTIL  123  GSVEEFQGQERSVILIS  21  NPKRFNVAVTRAKALL
Upf1p(S.c)    QVILVGDHQQL  31  LEVQYRMNPYLS  114  ASVDAFQGREKDYIILS  14  DPRRLNVGLTRAKYGL
     (S.p)    QVVLVGDHQQL  31  LVVQYRMHPCLS  114  ASVDAFQGREKDFIILS  14  DPRRLNVALTRAKYGV
Rent1(human)  QLILVGDHCQL  31  LQVQYRMHPALS  114  ASVDAFQGREKDFIILS  14  DPRRLNVALTRAKYGV
Rent1(mouse)  QLILVGDHCQL  31  LQVQYRMHPALS  114  ASVDAFQGREKDFIILS  14  DPRRLNVALTRARYGV
CONSENSUS II  QlILVGDH-QL      L-VQYRMHP-LS       ASVDAFQGREKDfIILS      DPRRLNVALTRAKYGv
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FIG. 3. Alignment of the sequence motifs common to members of the helicase superfamily I with sequences within recently defined putative helicases. The boundaries and designation of the conserved motifs is after ref. 21. The consensus I line is based upon defined members of superfamily I from prokaryotes and eukaryotes (viral proteins not included) and is modified from ref. 21. U, a bulky hydrophobic residue (Ile, Leu, Val, Met, Phe, Tyr, Trp); –, any residue. The number of residues between aligned segments is indicated. The consensus II line is derived from comparison of the specified segments of the putative new members of helicase superfamily I. Uppercase type denotes a residue in a given position that is identical in the majority of these proteins and lowercase type denotes a residue that is identical in at least half. Underlined residues in consensus I match the corresponding residue in consensus II.

consensus for a bipartate nuclear localization signal (27) and that is not conserved in Upf1p (Fig. 1).

The evolutionary pressure for maintenance of NMRD activity is unclear as none of the Upf proteins are required for vegetative growth in yeast. Likewise, although multiple *Caenorhabditis elegans* strains deficient in NMRD (*smg* mutants) show mild morphogenetic defects of genitalia and reduced brood sizes, their growth and development is otherwise normal (28). It has been postulated that the NMRD pathway protects the organism from deleterious dominant negative or gain-of-function effects that would be attributable to the truncated proteins expressed from nonsense alleles if the corresponding transcripts were stabilized (28). Although rent1 is expressed in all adult tissues that have been tested, it remains to be determined whether the observed tissue-specific variation in transcript abundance influences the regional efficiency of NMRD. If so, it is possible that spatial variation in rent1 expression modulates the tissue-specific pattern of phenotypic severity associated with selected inherited nonsense alleles.

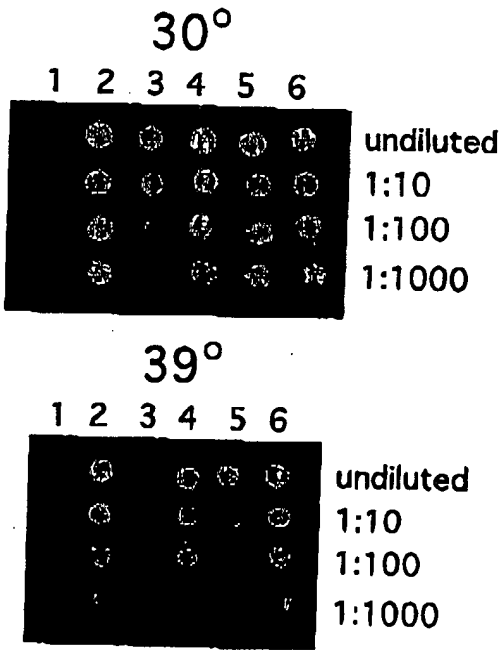

FIG. 4. Allosuppression growth assay of serial dilutions of strain PLY38 and multiple transformants at permissive (30°C) and nonpermissive (39°C) temperatures. Lanes: 1, untransformed; 2, transformed with p426MET25 (expression vector without insert); 3, pMET25-UPF (encoding wild-type Upf1p); 4, pMET25-y5′ (encoding Upf1p N terminus); 5, pMET25-CHIMERA (encoding Upf1p N and C termini flanking a large central region of rent1); 6, pMET25-RENT (encoding rent1). All strains grow at 30°C except untransformed PLY38 which is Ura–. At 39°C PLY38+pMET25-RNT grows as well as a strain containing vector alone, PLY38+pMBT25-UPF does not grow, and PLY38+pMET25-CHIMERA grows significantly slower. Three independent transformants were selected for each construct and tested for growth, with identical results (data not shown). An easily discernible intermediate growth phenotype was seen at 37°C (data not shown).

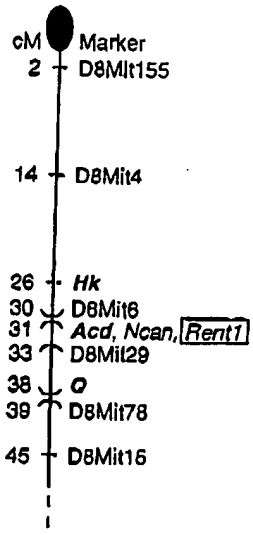

FIG. 5. Genetic map of murine chromosome 8 showing the relative positions of Mit microsatellite markers, mapped murine genes, the *Rent1* gene, and phenotypic murine markers (boldface type). Numbers indicate offset in centimorgans from the centromere (at the top). This map position of *Rent1* lies in a region that shows homology of synteny with human chromosome 19p13.2–p13.11. Screening of a somatic cell hybrid panel showed hybridization only to human chromosome 19-containing lines (data not shown).

The potential origins of nonsense transcripts include faulty transcription, inefficient splicing, and somatic or inherited mutations. Therefore, the phenotype resulting from a relative or global loss of NMRD function may be highly variable, depending upon the inherent mutability, environmental exposure, DNA repair capacity, and genetic background of the host. Although tumorigenic or accelerated aging phenotypes might be predicted to result from the somatic accumulation of a heterogeneous population of nonsense transcripts on an NMRD-deficient background, no apparently relevant phenotypes have been linked to the map positions for the murine and human genes encoding rent1 on chromosomes 8 and 19p13.2–p13.11, respectively (Fig. 5). Targeted disruption of RENT1 in mammalian cells and animal models should help to elucidate the biologic basis for complete evolutionary conservation of NMRD.

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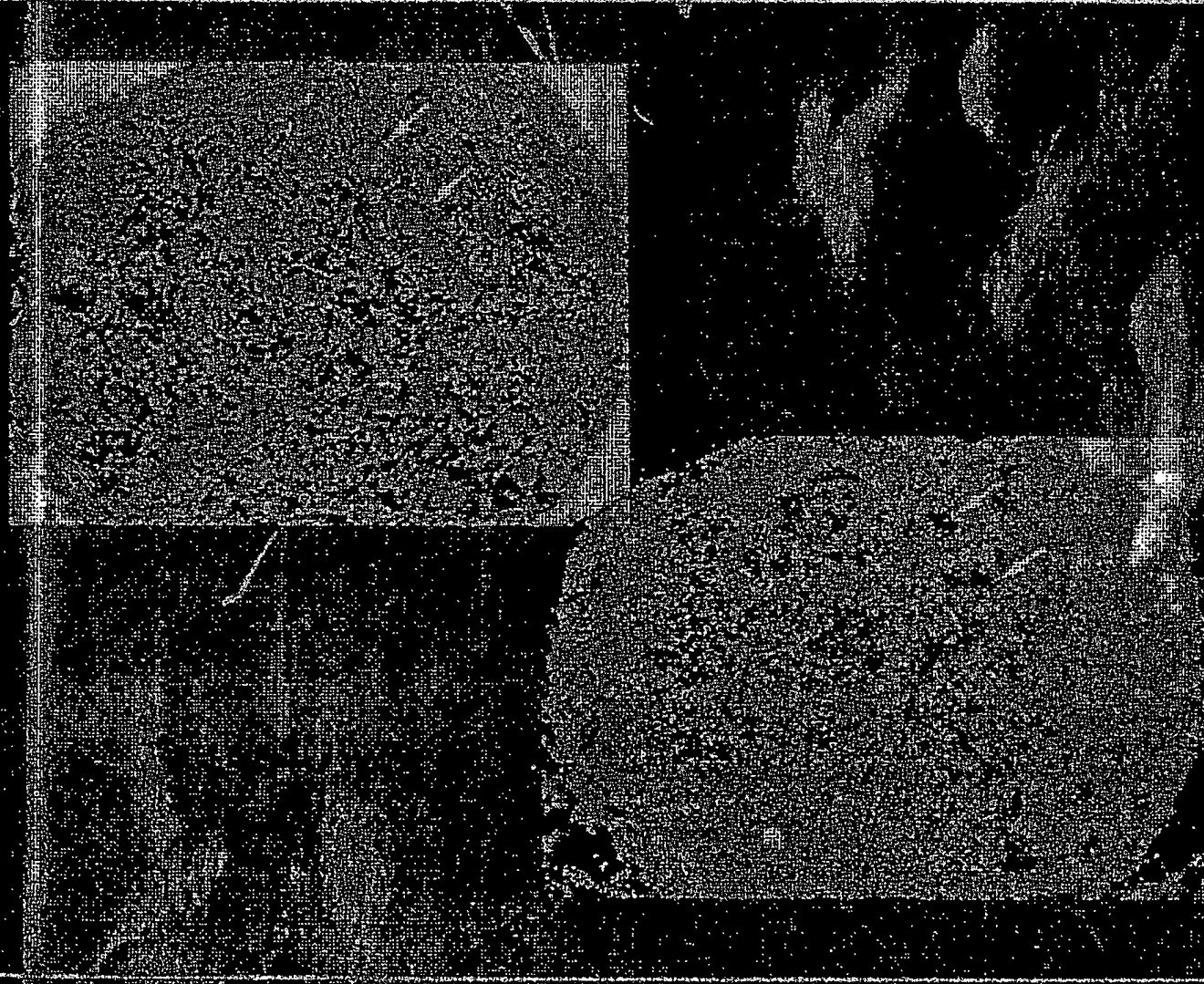

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Identification and characterization of genes that are required for the accelerated degradation of mRNAs containing a premature translational termination codon

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In both prokaryotes and eukaryotes nonsense mutations in a gene can enhance the decay rate of the mRNA transcribed from that gene, a phenomenon described as nonsense-mediated mRNA decay. In yeast, the products of the *UPF1* and *UPF3* genes are required for this decay pathway, and in this report we focus on the identification and characterization of additional factors required for rapid decay of nonsense-containing mRNAs. We present evidence that the product of the *UPF2* gene is a new factor involved in this decay pathway. Mutation of the *UPF2* gene or deletion of it from the chromosome resulted in stabilization of nonsense-containing mRNAs, whereas the decay of wild-type transcripts was not affected. The *UPF2* gene was isolated, and its transcript was characterized. Our results demonstrate that the *UPF2* gene encodes a putative 126.7-kD protein with an acidic region at its carboxyl terminus $(\text{-D-E})_n$ found in many nucleolar and transcriptional activator proteins. The *UPF2* transcript is 3600 nucleotides in length and contains an intron near its 5′ end. The *UPF2* gene is dispensable for vegetative growth, but *upf2Δ* strains were found to be more sensitive to the translational elongation inhibitor cycloheximide than *UPF2*$^+$. A genetic analysis of other alleles proposed to be involved in nonsense-mediated mRNA decay revealed that the *UPF2* gene is allelic to the previously identified *sua1* allele, a suppressor of an out-of-frame ATG insertion shown previously to reduce translational initiation from the normal ATG of the *CYC1* gene. In addition, we demonstrate that another suppressor of this *cyc1* mutation, *sua6*, is allelic to *upf3*, a previously identified lesion involved in nonsense-mediated mRNA decay.

[*Key Words*: RNA degradation; *UPF2*; nonsense mutations; translation]

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Differences in the decay rates of individual mRNAs can have profound effects on the overall levels of expression of specific genes. In eukaryotic cells the decay rates of mRNAs can differ from each other by >50-fold (for review, see Ross 1988; Peltz et al. 1991, 1993a; Peltz and Jacobson 1993; Sachs 1993). In addition, the decay rates of certain mRNAs are modulated depending on either the stage of the cell cycle, cell type or stage of differentiation and external factors such as hormonal levels, nutritional needs and environmental stresses (for review, see Cleveland and Yen 1989; Atwater et al. 1990; Peltz et al. 1991). The mechanisms that control these processes are largely unknown.

We have been studying mRNA turnover in the yeast *Saccharomyces cerevisiae*. Our results, as well as results from other laboratories, strongly indicate that mRNA turnover and translation are intimately linked processes and that understanding their relationship is critical to the understanding of mRNA decay (Stimac et al. 1984; Graves et al. 1987; Cleveland 1988; Gay et al. 1989a,b; Peltz et al. 1989; Parker and Jacobson 1990; Wisdom and Lee 1991; Bernstein et al. 1992; Laird-Offringa 1992; Peltz et al. 1992; Aharon and Schneider 1993; for review, see Pelsy and Lacroute 1984; Peltz et al. 1993a). One clear example of the relationship between translation and mRNA decay is the effect of nonsense mutations on the abundances and decay rates of mRNAs. In both prokaryotes and eukaryotes nonsense mutations in a gene can accelerate the decay rate of the mRNA transcribed from that gene 10- to 20-fold (Losson and Lacroute 1979; Maquat et al. 1981; Pelsy and Lacroute 1984;

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Baumann et al. 1985; Nilsson et al. 1987; Daar and Maquat 1988; Urlaub et al. 1989; Cheng et al. 1990; Gozalbo and Hohmann 1990; Leeds et al. 1991; Barker and Beemon 1991; Gaspar et al. 1991; Baserga and Benz 1992; Lim et al. 1992; Cheng and Maquat 1993; Peltz et al. 1993a). We use the term nonsense-mediated mRNA decay to describe this phenomenon (Peltz et al. 1993a). We have begun to identify the *cis*-acting sequences and *trans*-acting factors involved in this decay pathway (Leeds et al. 1991, 1992; Peltz et al. 1993a; for review, see Peltz et al. 1994).

A genetic screen for translational frameshift suppressors led to the identification of a class of mutant alleles termed *upf* (for up-frameshift; Culbertson et al. 1980; Leeds et al. 1992). Subsequent analysis of these alleles demonstrated that mutations in either the *UPF1* or *UPF3* genes led to the specific stabilization of nonsense-containing mRNAs (Leeds et al. 1991, 1992; Peltz et al. 1993a). For example, an amber codon that terminates translation of the *PGK1* transcript after 5% of its protein-coding region has been translated decreased its mRNA half-life from 60 min or greater to 3 min or less in a wild-type strain (Peltz et al. 1993a). In a *upf1*Δ strain the decay rate of the same mRNA is 60 min or greater (Peltz et al. 1993a). Similar observations have been found in strains containing mutations in the *UPF3* gene (K. Hagan and S.W. Peltz, unpubl.).

Experiments investigating the role of the *UPF2* gene in nonsense-mediated mRNA decay were ambiguous (Leeds et al. 1992). The results presented here identify and characterize the *UPF2* gene and demonstrate that similar to the *UPF1* and *UPF3* genes, the product of the *UPF2* gene is a necessary component for nonsense-mediated mRNA decay. Furthermore, we demonstrate that the previously identified *sua1* lesion (Hampsey et al. 1991; Pinto et al. 1992a) is allelic to *upf2* and that *sua6* is allelic to *upf3* (Pinto et al. 1992a,b).

Results

The abundance of nonsense-containing mRNAs is increased in strains harboring the upf2-1 *allele*

We wanted to determine whether the level of nonsense-containing mRNAs was increased in strains harboring a mutant *upf2* allele. The abundances of the *his4-38* mRNA and *CYH2* precursor were monitored in *upf1*$^-$, *upf2*$^-$, and wild-type strains (see Table 1). The *his4-38* allele is a single G insertion in the *HIS4* gene that generates a +1 frameshift and a UAA nonsense codon in the triplet adjacent to the insertion, resulting in rapid decay of this mRNA in wild-type cells (Fig. 1; Donahue et al. 1981; Leeds et al. 1991). The inefficiently spliced *CYH2* precursor is stabilized 5- to 10-fold in a *upf1*$^-$ strain because the intron sequences near the 5′ end contain an in-frame nonsense codon (He et al. 1993). RNAs from wild-type, *upf2*$^-$, and *upf1*$^-$ cells were isolated, and the abundances of the *CYH2* precursor and *his4-38* mRNA were determined by RNA blotting analysis involving hybridization to radioactive probes complementary to these RNAs. As shown in Figure 1, the abundances of the *CYH2* precursor and the *his4-38* transcript were low in wild-type cells and could be detected only after overdevelopment of the film but increased at least fivefold in both the *upf1*$^-$ and *upf2*$^-$ mutant strains. These results indicate that the product of the *UPF2* gene is involved in nonsense-mediated mRNA decay.

Isolation of the UPF2 *gene*

Strains harboring the *upf2*$^-$ allele were isolated on the basis of its ability to act as an allosuppressor of the *his4-38* frameshift mutation (Culbertson et al. 1980; Leeds et al. 1992). At 30°C, but not 37°C, the *his4-38* frameshift allele is suppressed by *SUF1-1*, which encodes a glycine tRNA capable of reading a 4-base codon (Mendenhall et al. 1987). Mutations in the *UPF* genes, including *UPF2*, allow cells harboring *his4-38 SUF1-1* to grow at 37°C. The wild-type *UPF2* gene was isolated by transformation of a yeast strain harboring the *upf2-1*, *his4-38*, and *SUF1-1* alleles with a yeast genomic library and screening for cells that could no longer grow on medium lacking histidine at 37°C. From 5000 colonies, nine colonies containing the single-copy plasmids were no longer capable of growing at 37°C (Fig. 2A). The strains harboring the nine plasmids identified above could overcome the allosuppressor phenotype of the *upf2* mutation. We next wanted to determine whether the loss of the allosuppressor phenotype corresponded with decreased abundance of the nonsense-containing mRNAs. Therefore, we determined whether the abundance of the *CYH2* precursor in cells harboring these plasmids was decreased compared with cells harboring only the *upf2* allele. RNAs were isolated from wild-type cells, *upf2*$^-$ cells, and *upf2*$^-$ cells harboring the plasmids identified above, and the abundance of the *CYH2* precursor was determined as described above. The results demonstrate that only one of the nine strains harboring the plasmids identified above has a reduced abundance of the *CYH2* precursor (Fig. 2B, lane 5). Strain YPF2-5 that had lost the plasmid after being plated on medium containing 5-FOA was now able to grow at the higher temperature (data not shown). The plasmid pYCpA5 was isolated and transformed again into a mutant *upf2* strain (*upf2-1 his4-38 SUF1-1*) and retested for inhibition of growth at 37°C. The plasmid that was transferred into the *upf2*$^-$ strain prevented the cells from growing at 37°C (data not shown). These results demonstrate that the modulation of the frameshift suppression was a consequence of the plasmid containing the yeast gene. The plasmid pYCpA5 harboring the putative *UPF2* gene (Fig. 2B, lane 5) was characterized further. The genes from the other plasmids that abrogate the translational effects of the *upf2* mutation have not been characterized.

A restriction map of the genomic 13.7-kb DNA fragment contained in the plasmid pYCpA5 harboring the putative *UPF2* gene was constructed (Fig. 3A). Plasmid subclones of the genomic DNA were prepared (Fig. 3B), and their ability to affect the allosuppressor phenotype (data not shown) and the abundance of the *CYH2* precur-

Table 1. *Strains used in this study*

Strain	Genotype	Source, reference, or derivation
PLY36	*MATα his4-38 SUF1-1 upf1-2 ura3-52 met14*	Leeds et al. (1991), (1992)
PLY18	*MATa his4-38 SUF1-1 ura3-52 trp1-1 leu2-3*	Leeds et al. (1991), (1992)
PLY136	*MATa his4-38 SUF1-1 upf2-1 ura3-52*	Leeds et al. (1991), (1992)
YGC110	*MATα his4-38 SUF1-1 upf2-1 ura3-52*	this study
PLY139	*MATa his4-38 SUF1-1 upf3-1 ura3-52*	Leeds et al. (1991), (1992)
YGC10$^-$	*MATα his4-38 SUF1-1 upf1-2 ura3-52 met14 [YCplac33]*	PLY36 derivative
YGC10$^+$	*MATα his4-38 SUF1-1 upf1-2 ura3-52 met14 [YCp33UPF1]*	PLY36 derivative
YGC12$^-$	*MATa his4-38 SUF1-1 upf2-1 ura3-52 [YCp50]*	PLY136 derivative
YGC112	*MATa his4-38 SUF1-1 UPF2::URA3 ura3-52 trp1-1 leu2-3*	this study
RY262	*MATα rpb1-1 his4-519 ura3-52*	Peltz et al. (1993a)
YGC14$^+$	*MATα rpb1-1 his4-519 ura3-52 [YCplac33]*	RY262 derivative
YGC114	*MATα rpb1-1 his4-519 UPF2::URA3 ura3-52*	this study
YGC116	*MATα rpb1-1 his4-519 UPF1::hisG UPF2::URA3 ura3-52*	this study
Y52$^-$	*MATα rpb1-1 his4-519 UPF1::hisG ura3-52*	this study
YGC16$^-$	*MATα rpb1-1 his4-519 UPF1::hisG ura3-52 [YCplac33]*	Y52$^-$ derivative
RL92	*MATa prp2 leu2-3 leu2-112 ura3-52*	Beate Schwer's laboratory
YGC118	*MATa his4-38 SUF1-1 upf3-1 UPF2::URA3 ura3-52*	this study
YGC120	*MATα his4-38 SUF1-1 upf1-2 UPF2::URA3 ura3-52 met14*	this study
YGC112EP2	*MATa his4-38 SUF1-1 UPF2::URA3 ura3-52 trp1-1 leu2-3 [YEpUPF2]*	YGC112 derivative
T16	*MATα cyc1-5000 cyc7-67 ura3-52 leu2-3,112 cyh2 SUA$^+$*	Hampsey et al. (1991); Pinto et al. (1992)
YIP15-4A	*MATα cyc1-362 arg4-17 leu2-3,112 sua1*	Hampsey et al. (1991); Pinto et al. (1992)
YIP15-4B	*MATα cyc362 his3-Δ1 ura3-52 sua2*	Hampsey et al. (1991); Pinto et al. (1992)
YIP15-4D	*MATa cyc1-362 arg4-17 ura3-52 sua1 sua2*	Hampsey et al. (1991); Pinto et al. (1992)
YIP13-11A	*MATα cyc1-362 arg4-17 his3-Δ1 ura3-52 sua3*	Hampsey et al. (1991); Pinto et al. (1992)
YIP16-4D	*MATa cyc1-362 his3-Δ1 ura3-52 sua4*	Hampsey et al. (1991); Pinto et al. (1992)
YJN192	*MATα cyc1-1019 cyc7-67 ura3-52 leu2-3,112 cyh2^r sua5-1*	Hampsey et al. (1991); Pinto et al. (1992)
YJN-8A	*MATα cyc1-362 ura3-52 leu1-12 his5-2 (trp5!) sua6*	Hampsey et al. (1991); Pinto et al. (1992)

sor was analyzed (Fig. 3C). A plasmid harboring the genomic fragment containing a deletion between the two *Bam*HI DNA restriction sites increased the abundance of the *CYH2* precursor (Fig. 3B,C, pYCpAΔB), indicating that the putative *UPF2* gene was located in this region of the DNA fragment. Plasmid subclones that contained either a 7.1-kb DNA fragment (Fig. 3B,C, pYCpAp7.1) or a 5.4-kb DNA fragment (Fig. 3B,C, pYCpAB5.4) were able to decrease the abundance of the *CYH2* precursor in a *upf2*$^-$ strain (Fig. 3C). DNA subclones that contained smaller regions of the yeast genomic DNA fragment failed to complement the *upf2* mutant strain as determined by the ratio of the *CYH2* precursor to *CYH2* mRNA (Fig. 3B,C, i.e., pYCpA3.5, pYCpA5.0, pYCpA5.35, and pYCpAX6.6).

DNA sequence analysis of the UPF2 *gene*

The sequence of the DNA fragment harboring the putative *UPF2* gene in plasmid pYCpAp7.1 was determined. The sequence of a 5.015-kb DNA fragment from 100 bp upstream of the first *Cla*I site to ~1400 nucleotides downstream of the *Eco*RI site was determined (Fig. 4). Our initial inspection of the sequence identified a 1091-amino-acid open reading frame (ORF), but subsequent analysis demonstrated a perfect splice site branchpoint consensus sequence 33 bp upstream of the ORF and the consensus acceptor site (Fig. 4, 5′-TACTAAC-3′, between 65 and 71 bp; for review, see Rymond and Rosbash 1993). The location of the splice donor site could not be identified by computer analysis. Subsequent analysis of the *UPF2* mRNA indicated that the transcript contained a short intron very near the 5′ end and encoded a 1089-amino-acid protein (Figs. 4 and 5; see below). These findings are consistent with the observation that in the yeast *S. cerevisiae* the introns are usually found near the 5′ end of the mRNA.

The predicted peptide sequence of the Upf2 protein (Upf2p) was used to search the SWISSPROT and nonredundant protein sequence data bases by use of the GCG program. With the exception of its carboxyl terminus, Upf2p had no significant homology to other proteins.

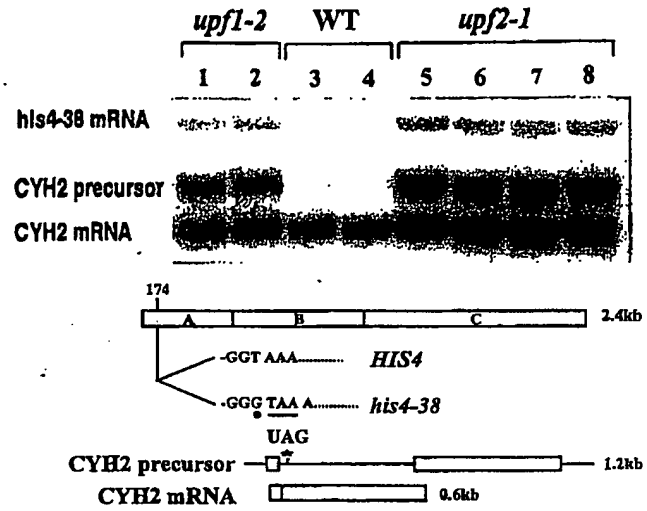

Figure 1. The *CYH2* precursor and *his4-38* mRNA accumulate in strains harboring the *upf2-1* allele. mRNA abundances for the *his4-38* mRNA, *CYH2* precursor and *CYH2* mRNA were determined in wild-type, *upf1*$^-$, and *upf2*$^-$ yeast strains by RNA blot analysis of RNAs. Total RNA (20 μg) was loaded on each lane. The RNA blot was hybridized with radiolabeled *HIS4* and *CYH2* probes. (Lanes *1–2*) Yeast strain YGC10$^-$ (*upf1*$^-$); (lanes *3–4*) YGC10$^+$ (wild-type (WT)); (lanes *5–8*) YGC12$^-$ (*upf2*$^-$). (*Bottom*) Schematic representation of the *CYH2* precursor and its spliced product and *his4-38* allele.

The carboxyl terminus of the Upf2p (amino acids 838–995) were highly homologous to a group of nucleolar or nuclear acidic proteins (with an identity score of 30% and a similarity score of 90%), including nucleolin (Maridor et al.1990; Srivastova et al. 1990), nucleolar phosphoprotein B23 (Chang et al. 1988), nucleolar transcription factor upstream binding factor (UBF) (O'Mahony et al. 1992; Voit et al. 1992), and yeast RNA polymerase III subunit RPC31 (Mosrin et al. 1990). The first three nucleolar proteins are known to be critical factors in ribosomal biogenesis. Within this region of homology all of these proteins have a conserved acidic amino acid stretch that is considered to be a casein kinase II phosphorylation site and is thought to be important for the function of these proteins (Chan et al. 1986; Chang et al. 1988; O'Mahony et al. 1992; Voit et al. 1992). The acidic region of the yeast RNA polymerase III gene has been demonstrated to be essential for its *trans*-activation function (Mosrin et al. 1990; O'Mahony et al. 1992; Voit et al. 1992). Although the role of the acidic region in the Upf2p is not known, on the basis of previous studies, we suggest that its role is probably involved in regulating the phosphorylation status of the protein which, in turn, is in some way necessary for maintenance of the protein–protein interactions. A search for other protein motifs by use of the PROSITE data base (v. 2/94) did not reveal any other known conserved motifs.

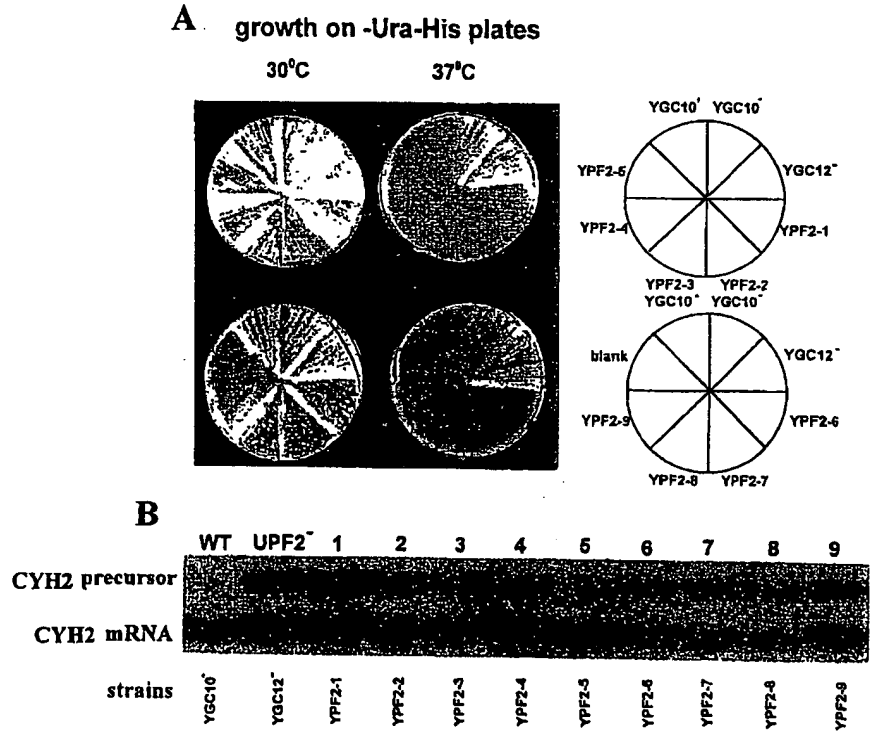

Figure 2. Isolation of a plasmid harboring the *UPF2* gene. The yeast strain PLY136 (*MATa his4-38 SUF1-1 upf2-1 ura3-52*) was transformed with a yeast genomic library, and cells harboring plasmids were selected by plating on medium lacking uracil. Cells harboring the putative *UPF2* gene were isolated by a screen (by replica plating) for cells that could no longer suppress the *his4-38* frameshift allele and therefore could not grow on medium lacking uracil and histidine at 37°C (as described in Materials and methods). (*A*) Growth phenotype of *upf2-1* strains harboring plasmids containing the putative *UPF2* gene. The strains used are YGC10$^+$ (*UPF1*$^+$); YGC10$^-$ (*upf1*$^-$); YGC12$^-$ (*upf2*$^-$); YPF2-*n* refers YGC12$^-$ lacking pYCP50 but harboring plasmids that contain the putative *UPF2* gene. The transformants were replica-plated onto medium lacking histidine and uracil and grown at either 30°C and 37°C for 4 days. (*B*) The abundance of the *CYH2* precursor and mRNA were assayed in strains harboring plasmids containing the putative *UPF2* gene. RNAs were isolated from strains YPF2-1 to YPF2-9 (lanes *1–9*, respectively), and from wild-type (YGC10$^+$) and *upf2*$^-$ (YGC12$^-$) strains and RNA blots were prepared and hybridized with a *CYH2* probe as described in Materials and methods. The *CYH2* precursor and *CYH2* mRNA are indicated (see Fig. 1. for a schematic of the *CYH2* precursor and *CYH2* mRNA).

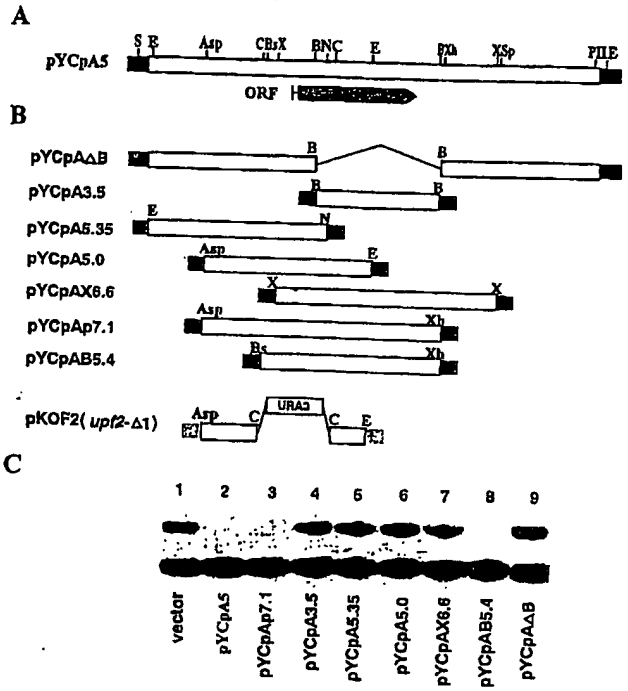

Figure 3. Identification of the putative *UPF2* gene. (*A*) A restriction map of yeast genomic DNA harboring the putative *UPF2* gene. The open reading frame (ORF) of the putative *UPF2* gene is shown. The shaded region in the schematic represents a portion of the pYCp50 vector. The restriction enzymes used were (S) *Sal*I; (E) *Eco*RI; (Asp) *Asp*718; (C) *Cla*I; (Bs) *Bst*XI; (X) *Xba*I; (B) *Bam*HI; (N) *Nru*I; (Xh) *Xho*I; (Sp) *Sph*I; (PII) *Pvu*II. (*B*) Determination of the region of the yeast genomic fragment harboring the putative *UPF2* gene and construction of a *upf2* disruption plasmid. The DNA from the yeast genomic fragment was cleaved with the restriction enzymes shown in the figure and various subclones were prepared by insertion of these DNA fragments into pYCplac33. plasmid pKOF2 was made to delete the *UPF2* gene from the yeast chromosome and was constructed as described in Materials and methods. (*C*) Identification of the DNA fragment harboring the putative *UPF2* gene. The plasmids described in *B* were transformed into PLY136 and the abundance of the *CYH2* precursor and mRNA were determined as described in Fig. 1.

The gene that complements the upf2-1 *allele encodes the* UPF2 *gene*

To determine whether the gene that we have isolated encodes the *UPF2* gene, a genomic disruption of the *UPF2* gene was constructed (Fig. 3B, pKOF2). Haploid yeast cells harboring the putative *UPF2* gene disruption were constructed and confirmed by Southern blot analysis (see Materials and methods). The viability of the haploid strain demonstrates that the putative *UPF2* gene was not essential for vegetative growth (Table 2, items 1 and 2). To determine whether the genomic disruption was able to complement the *upf2-1* allele, strain YGC112 containing the *his4-38 SUF1-1* alleles and the genomic disruption of the putative *UPF2* gene was crossed with strains harboring the *his4-38* and *SUF1-1* alleles that contained either the *upf1-2* or *upf2-1* alleles (Table 2, items 3 and 4). The complementation of the mutant alleles were determined by replica plating of the diploids onto media lacking histidine at 30°C or 37°C. Complementation occurs if the diploids are not able to grow at 37°C on media lacking histidine. A *upf1-2* strain was able to complement a strain harboring the disruption of the putative *upf2* gene (Table 2, item 3), whereas the putative *upf2*Δ strain was unable to complement the strain harboring the *upf2-1* allele (Table 2, item 4). In addition, the *CYH2* precursor abundance was high in the diploid prepared from a cross between the *upf2-1* and *upf2*Δ (Table 2, item 4), whereas the cross between *upf1-2* and *upf2*Δ reduced the intron-containing RNA fivefold (Table 2, item 3). These results demonstrate that the gene we have identified encodes the *UPF2* gene.

Analysis of the UPF2 *transcript*

We have analyzed the structure of the *UPF2* transcript by a variety of techniques. The *UPF2* transcript was visualized by RNA blotting analyses of RNAs isolated from *upf2*Δ cells harboring either a centromere or 2μ plasmid containing the *UPF2* gene or a plasmid lacking the *UPF2* gene. The results demonstrated that the *UPF2* mRNA was ~3600 nucleotides in length and was absent from a *upf2*Δ strain (Fig. 5A). The *UPF2* gene contains splicing consensus elements near the putative 5′ end of the *UPF2* gene (Fig. 4). A PCR strategy was employed to identify the exon/intron boundaries of the *UPF2* transcript. Total RNA was isolated, and cDNA from the RNA was prepared by reverse transcription. DNA primers that traversed the putative exon/intron boundaries were used in a PCR. A DNA fragment corresponding to the cDNA fragment from a spliced mRNA was isolated and sequenced (Fig. 5B,C). The DNA fragment described above was absent if the RNAs utilized in the reaction were prepared from a temperature-sensitive splicing-defective *prp2* strain (Table 1, RL92) shifted to the nonpermissive temperature (data not shown) or if reverse transcriptase was left out of the reaction mixture (Fig. 5B, lane 2). The 390-bp DNA fragment was sequenced, and the results indicate that the *UPF2* mRNA is spliced and that the splice site donor and splice site acceptor is located at 7 and 120 bp, respectively (Figs. 4 and 5). The splicing branchpoint is situated at 65–71 bp (Fig. 4).

The transcription start site of the *UPF2* mRNA was determined by primer extension analysis. Total RNA was isolated and a ^{32}P-labeled oligonucleotide complementary to the RNA in the second exon (Fig. 4) was used to prime a reverse transcriptase reaction. The results of the experiment demonstrate that the major transcription start site is located at 68 nucleotides 5′ of the translation start site (Figs. 4, 5D).

SUA1, *which is allelic to* UPF2, *and* SUA6, *which is allelic to* UPF3, *are* trans*-acting factors in the nonsense-mediated mRNA decay pathway*

Strains harboring the *cyc1-362* allele are deficient in iso-1-cytochrome *c* activity as a consequence of a mutation

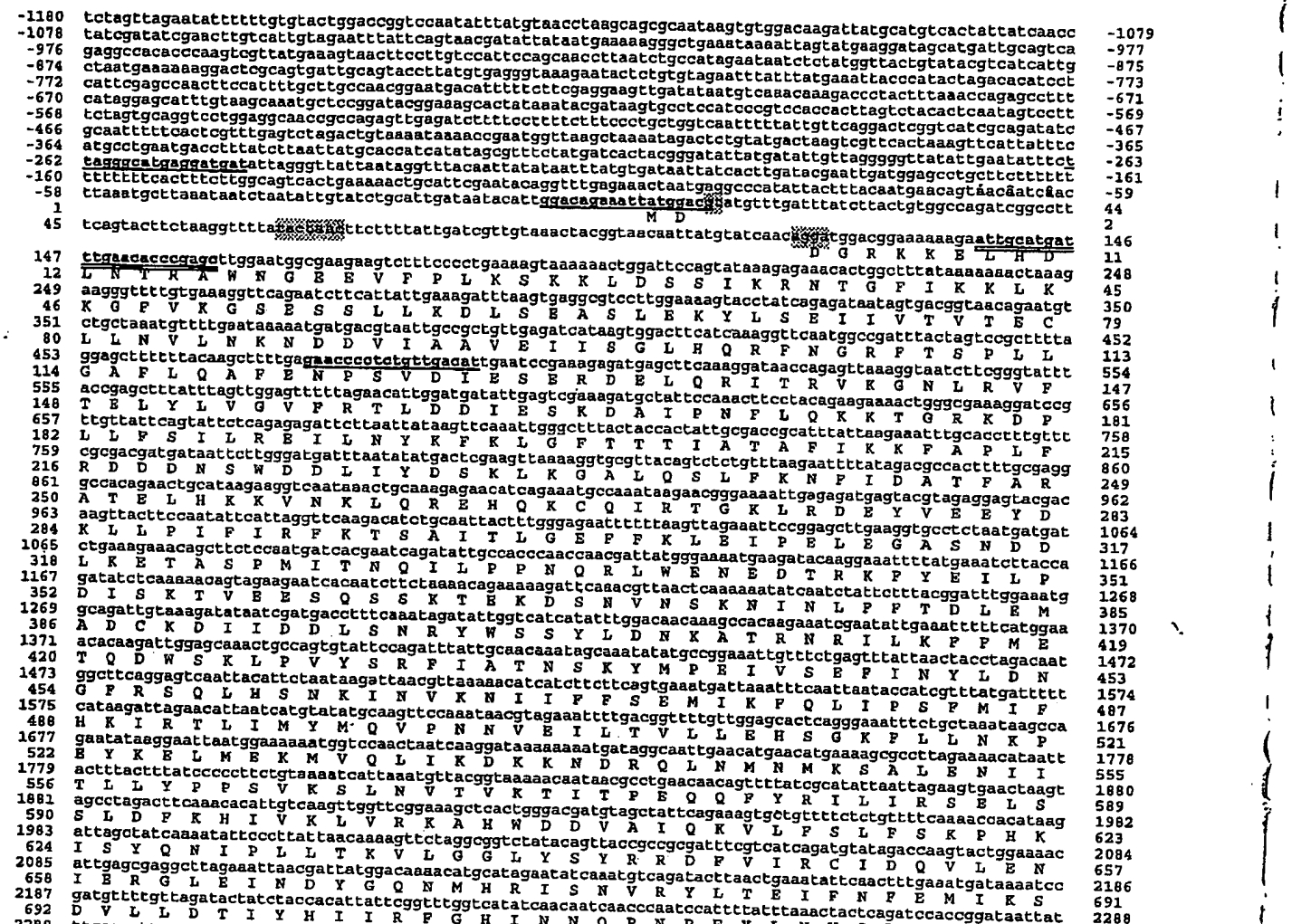

Figure 4. Sequence of the *UPF2* gene. The *UPF2* gene was sequenced as described in Materials and methods. The predicted amino acid sequence is shown in the single-letter code. Shaded nucleotides represent the consensus splice site sequence. The oligonucleotides used in isolation of the *UPF2* cDNA by PCR are bold and underlined. Boldface double-underlined nucleotides are the oligonucleotides for primer extension. Nucleotides marked by open circles or carets indicate the major or minor transcription start sites, respectively. The bold amino acid sequences (838–995) make up the acidic amino acid-rich region homologous to nucleolin.

resulting in an out-of-frame ATG codon upstream of the CYC1 protein-coding region (Stiles et al. 1981). The *cyc1-362* mutation is therefore analogous to an amino-terminal nonsense mutation. Suppressors of the *cyc1-*

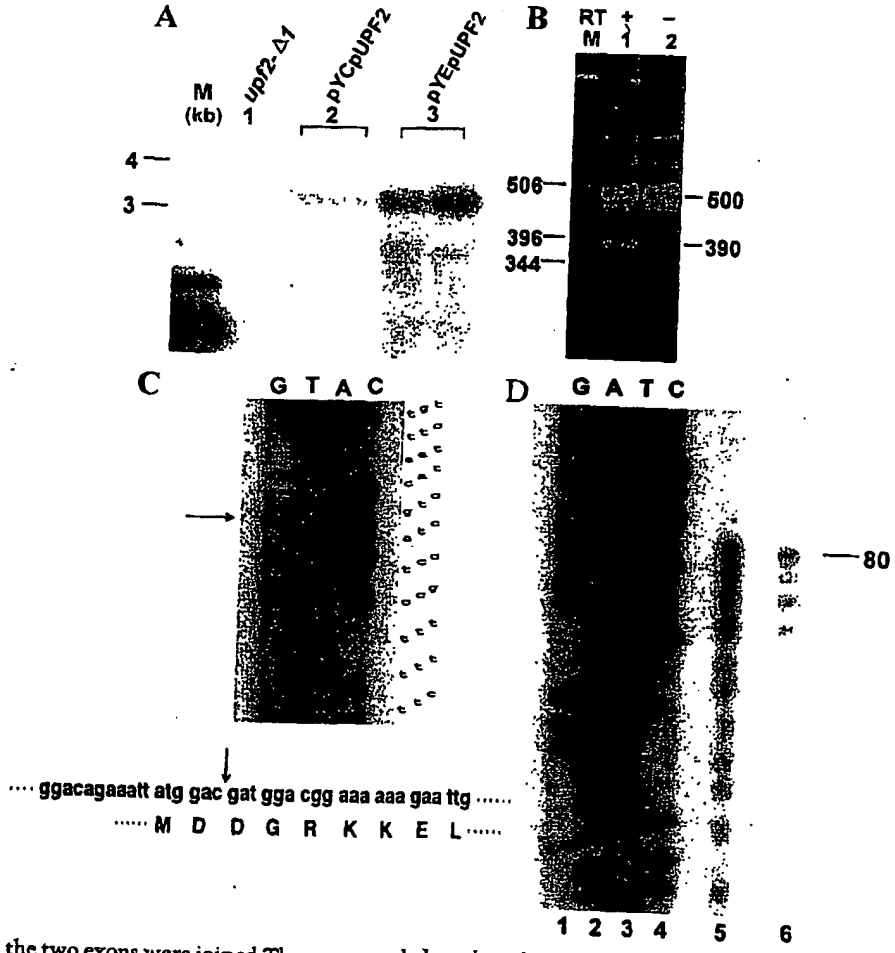

Figure 5. Analysis of the *UPF2* mRNA. (*A*) RNA blotting analysis of the *UPF2* transcript. RNAs were prepared from yeast strain YGC112 (*upf2Δ*; lane *1*), from yeast strain PLY136 containing the single-copy plasmid YCpAp7.1 (lane *2*) or strain YGC112 containing the high copy plasmid YEpAp7.1 (lane *3*); an RNA blot was prepared as described above, and the membrane was hybridized with radiolabeled a 1.7-kb *Bam*HI–*Eco*RI DNA fragment harboring the *UPF2* open reading frame (see Fig. 3). (*B,C*) Identification of the intron–exon junction of *UPF2* RNA. Total yeast RNA (30 μg) from PLY18 containing the high copy plasmid pYEpAp7.1 was reverse transcribed by use of hexamers as described in Materials and methods. The product of reverse transcription was subjected to PCR with primers b and c that we hypothesized to be near the 5′ and 3′ splice junctions (see Materials and methods). The PCR product was electrophoresed on a 1.5% agarose gel and is shown in *B*. Marker lane (M), 1-kb marker. (Lane *1*) The PCR product in which the reaction mixture contained reverse transcriptase (RT); (lane *2*) the PCR product in which the reaction mixture did not contain RT. (*C*) DNA sequence analysis of the 390-bp PCR product. The 390-bp DNA fragment from PCR was isolated and sequenced as described in Materials and methods. The arrow at *left* depicts the region where the two exons were joined. The sequence below the gel represents the DNA and protein sequences at the exon joining region. (*D*) Primer extension analysis of the *UPF2* transcript. Total yeast RNA prepared from strain YGC112 harboring pYEpAp7.1 plasmid was used in a reverse transcription reaction (see Materials and methods). Lanes *1–4* are the results of DNA sequencing reactions by use of the same primer as for the primer extension analysis (Materials and methods). (Lane *5*) Fifty micrograms of RNA in the reaction; (lane *6*) 30 μg of RNA used in the reaction. The mark at *right* indicates the length of the reverse transcription product. See Fig. 4 for the location of the transcription start site in the *UPF2* gene.

362 (called *sua* for suppressor of upstream ATG) have been isolated (*sua1–sua8*) and *sua7* and *sua8* have been characterized and shown to affect transcription start site selection (Hampsey et al. 1991; Pinto et al. 1992a,b). The mechanism of suppression for the other *sua* alleles has not been determined. In our search for other *trans*-acting factors involved in nonsense-mediated mRNA decay, we hypothesized that a subclass of the *sua* alleles would be involved in this decay pathway. Therefore, we asked whether any of the *sua* alleles affect the abundance of nonsense-containing mRNAs and whether they are allelic to the previously identified *upf* alleles. As described above, we utilized the abundance of the *CYH2* precursor as an assay to determine whether the *sua* alleles affected the activity of the nonsense-mediated mRNA decay pathway. RNAs were isolated from strains harboring either wild-type or mutant *sua* alleles, and the *CYH2* precursor and mRNA abundances were determined by RNA blotting analysis. The abundance of the *CYH2* precursor relative to the *CYH2* mRNA abundance was increased in strains containing either *sua1* or *sua6* allele but not in strains harboring the other *sua* alleles (Fig. 6A). These results indicate that the products from the *SUA1* and *SUA6* genes are involved in nonsense-mediated mRNA decay.

Complementation analysis was performed to determine whether the *sua1* or *sua6* mutations were allelic to any of the *upf* alleles. Strains harboring either the *sua1* or *sua6* alleles were transformed with centromere-based plasmids harboring either the *UPF1* or *UPF2* gene, and the abundance of the *CYH2* precursor was determined and compared with the RNA abundance of the *CYH2* precursor in a wild-type *SUA*$^+$ strain. The abundance of the *CYH2* precursor decreased fivefold when a strain harboring the *sua1* allele was transformed with a plasmid harboring the *UPF2* gene (Fig. 6B, lanes 3, 4). In addition,

Table 2. *Growth characteristics and CYH2 precursor and mRNA levels in crosses between strains harboring upf2Δ with either upf2-1 or upf1-2 alleles*

Item number	Strain	Growth[a] 30°C	Growth[a] 37°C	*CYH2* precursor/ *CYH2* mRNA[b]
1	YGC112 (*upf2Δ*)	+	+	0.54
2	YGC114 (*upf2Δ*)	+	N.D.	0.64
3	YGC112 × PLY36 (*upf2Δ* × *upf1-2*)	+	−	0.05
4	YGC112 × PLY136 (*upf2Δ* × *upf2-1*)	+	+	0.40

[a]Growth was determined on media lacking histidine. Yeast strains were grown on YPD media and replica plated onto yeast medium lacking histidine. The growth was followed for 4 days. (+) Strains able to grow on media lacking histidine; (−) cells unable to grow on this medium. (N.D.) Not determined.
[b]The ratios of the levels of the *CYH2* precursor to *CYH2* mRNA were determined as described in Fig. 1 and in Materials and methods. The *CYH2* precursor and mRNAs were quantitated by use of a Bio-Rad densitometer.

a *upf2Δ* strain failed to complement a strain harboring a *sua1* allele as determined by the high *CYH2* precursor level in the diploid cell (Fig. 6B, lanes 13, 14), indicating that *SUA1* and *UPF2* are the same gene. The *sua6* allele was not complemented by any of the identified *UPF* genes, indicating that it was not *UPF1* or *UPF2* (Fig. 6B, lanes 7–12). Crossing the *sua6* mutant cell with a strain harboring *upf3-1* allele demonstrated that the abundance of the *CYH2* precursor was high in a *sua6/upf3* diploid cell. Tetrad analysis from seven *sua6/upf3* diploid strains also confirmed that the levels of *CYH2* precursor in all spores were the same as observed in the parental *upf3* or *sua6* strain (one example of these results is shown in Fig. 6B, bottom), indicating that *SUA6* is the same gene as *UPF3*.

Deletion of the UPF2 gene from the yeast chromosome stabilizes nonsense-containing mRNAs without affecting the decay of wild-type transcripts

We next determined whether the high steady-state levels of nonsense-containing mRNAs in a *upf2Δ* strain resulted from stabilizing their transcripts. The mRNA decay rates of wild-type and nonsense-containing mRNAs were determined in *UPF2*$^+$ and *upf2Δ* strains that also harbored the temperature-sensitive allele of RNA polymerase II (*rpb1-1*). The decay rates of the wild-type and nonsense-containing mRNAs were determined by RNA blotting analyses of RNA isolated at different times after inhibition of transcription by a shift of the culture to the nonpermissive temperature (36°C). The results of these experiments demonstrate that the nonsense-containing *his4-519* and *CYH2* precursor RNAs were stabilized 10- and 8-fold, respectively (Fig. 7; Table 3) compared with *UPF2*$^+$ cells, whereas the half-lives of the wild-type *CYH2*, *MATα1*, *LEU2*, and *TIF4631*, were the same in either *UPF2*$^+$ or *UPF2Δ* strains (Table 3). These results demonstrate that the *UPF2* gene is involved in nonsense-mediated mRNA decay.

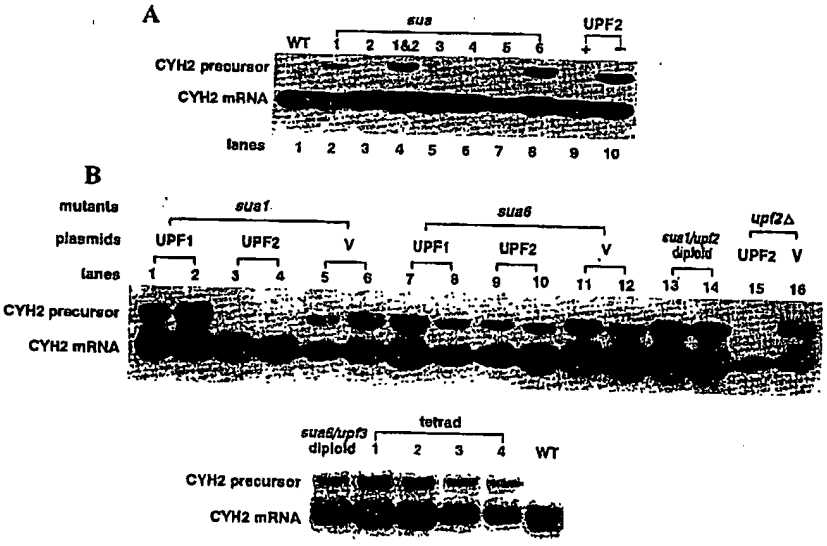

Figure 6. *sua1* and *sua6* affect the abundance of the *CYH2* precursor. (*A*) Wild-type and *sua*-containing strains were grown. RNAs were prepared and the abundances of *CYH2* precursor and mRNA were assayed by RNA blotting as described in Fig. 1. The results from *sua* alleles are shown in lanes *2–8* (the captions at *top* describe the *sua* allele tested). Cells harboring the wild-type *UPF2* (lanes *1*, *9*) or the *upf2-1* allele (lane *10*) are shown as controls. (*B*) Determination of whether *sua1* or *sua6* is allelic to the *UPF* alleles. Plasmid pYCplac33 harboring either the *UPF1* or the *UPF2* gene, or just vector, were transformed individually into strains harboring either *sua1* or *sua6*. The strains were grown in medium lacking uracil, RNAs were prepared, and the abundance of *CYH2* precursor and mRNA was assayed by RNA blotting as described in Fig. 1. Lanes *1–6* are results from strains harboring one of the various plasmids described. Lanes *13–14* are results from a *sua1* diploid yeast strain prepared by crossing *sua1* strain with the *upf2Δ* strain (see Material and methods for description of the strain construction). Lanes *15–16* are results from *upf2Δ* strain harboring either wild-type *UPF2* plasmid or vector alone, respectively. Lanes *7–12* show the results from *sua6* strain transformed with different plasmids as described. (*Bottom*) A depiction of results from an RNA blotting assay (hybridized with a radioactive *CYH2* probe) with RNAs isolated from a *sua6/upf3* diploid cell (which was prepared from a cross between *sua6* strain and *upf3-1* strain). In addition, a total of seven tetrads were analyzed, and one example of RNA analysis from the spores of a single tetrad after sporulation of the diploid cell is shown here.

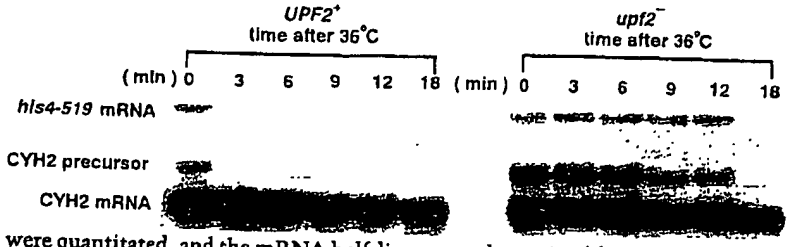

Figure 7. Decay rates of the nonsense-containing *his4-519* mRNA and *CYH2* precursor and mRNA as determined in *UPF2*$^+$ and *upf2*$^-$ strains. mRNA decay rates of the *his4-519*, *CYH2* precursor and mRNA were determined in *UPF2*$^+$ (RY262) and *upf2*$^-$ (YGC114) strains by a temperature-shift experiment as described in Materials and methods. The RNA blots were hybridized with *HIS4* and *CYH2* probes. The results shown were quantitated, and the mRNA half-lives were determined from the semi-log plot of the percent of mRNA remaining versus the time after the temperature was shifted.

Strains harboring multiple mutations in the UPF *genes do not affect cell viability or increase the stability of nonsense-containing mRNAs*

We wanted to determine whether a strain harboring a *upf2Δ* in combination with other *upf* alleles would either affect cell viability or exacerbate the stabilization of nonsense-containing mRNAs. Strains harboring *upf1Δ* *upf2Δ* alleles and *upf2Δ* *upf3-1* alleles were constructed and were shown to be viable with no apparent growth defect (Table 1; data not shown). As described above, we utilized the abundance of the *CYH2* precursor as an assay to determine the effects of these mutations on the activity of the nonsense-mediated mRNA decay pathway. RNAs from *upf1Δ*, *upf2Δ*, *upf3-1*, *upf1Δ* *upf2Δ*, and *upf2Δ* *upf3-1* strains were isolated, and the *CYH2* precursor and mRNA abundances were determined by RNA blotting analysis. A summary of these results is shown in Table 4. The results indicate that strains harboring pairwise combinations of the *upf2* allele with the other *upf* alleles were viable but did not increase the abundance of the *CYH2* precursor further in these cells (Table 4).

Strains harboring a genomic disruption of the UPF2 *gene were slightly sensitive to cycloheximide*

Previous experiments have demonstrated that, compared to wild-type cells, *upf1Δ* strains are sensitive to the translation elongation inhibitor cycloheximide but not to paromomycin (Leeds et al. 1992), a drug that decreases translational fidelity during elongation (Palmer et al. 1979; Singh et al. 1979). We wanted to determine the drug sensitivity of strains harboring a deletion of the *UPF2* gene to these drugs. Isogenic wild-type, *upf1Δ*, *upf2Δ*, or *upf1Δ* *upf2Δ* strains were grown, and discs containing either paromomycin or cycloheximide were placed onto the plate and the growth of these cells were monitored. By comparing the zone of growth inhibition around the disc containing the drug, the antibiotic sensitivity of these strains can be assessed. We found that *upf1Δ*, *upf2Δ*, and *upf1Δ* *upf2Δ* strains were slightly more sensitive to cycloheximide than wild-type cells (the diameter of the inhibited growth zone was 3.72 cm for wild-type, 4.26 cm for *upf1Δ*, 4.32 cm for *upf2Δ*, and 4.23 cm for *upf1Δ* *upf2Δ*) but have the same sensitivities to paromomycin as a wild-type *UPF1*$^+$ *UPF2*$^+$ strain (the diameter of the inhibited growth zone was 1.10 cm for wild-type, 1.09 cm for *upf1Δ*, 1.07 cm for *upf2Δ*, and 1.10 cm for *upf1Δ* *upf2Δ*). The sensitivity of the *upf1Δ* *upf2Δ* strain to cycloheximide was not greater than strains harboring either of the individual mutant *upf* alleles. These results suggest that the product of the *UPF2* gene, either directly or indirectly, may alter ribosome structure.

Discussion

A large body of experiments has demonstrated a strong relationship between the processes of translation and mRNA turnover. *Cis*-acting sequences that promote instability of mRNAs have been identified in the protein-coding regions as well as in 3′-untranslated regions of transcripts, and recent results have demonstrated that on-going translation is required for these elements to promote mRNA decay (Graves et al. 1987; Cleveland 1988; Gay et al. 1989a,b; Shyu et al. 1989; Wisdom and Lee 1991; Heaton et al. 1992; Herrick and Jacobson 1992; Laird-Offringa 1992; Peltz et al. 1992, 1993; Aharon and Schneider 1993; Caponigro et al. 1993; Herrick and Ross 1994). The role of translation in determining mRNA decay rates is not indirect, and at least for a subset of instability elements, the sequences that promote mRNA turnover must be actively translated to induce mRNA decay (Graves et al. 1987; Cleveland 1988; Gay et al. 1989a; Parker and Jacobson 1990; Wisdom and Lee 1991;

Table 3. *mRNA decay rates in wild-type and* upf2Δ *strains*

mRNA	$t_{1/2}$ (min) *UPF2*$^+$	*upf2Δ*
CYH2 precursor	1.0	7.6
his4-519	1.2	13.4
CYH2 mRNA	48	54
MATα1	3.5	3.8
LEU2	15.8	15.1
TIF4631	15.6	15.6

The decay rates for these mRNAs were determined as described in Fig. 7 and in Materials and methods.

Table 4. *Multiple mutations in the UPF genes are not additive in affecting the abundance of the* CYH2 *precursor*

Strain	Genotype	*CYH2* precursor/ *CYH2* mRNA
YGC14$^+$	*UPF1*$^+$ *UPF2*$^+$ *UPF3*$^+$	0.05
Y52$^-$	*upf1Δ*	0.31
YGC114	*upf2Δ*	0.52
PLY139	*upf3-1*	0.43
YGC116	*upf1Δ upf2Δ*	0.38
YGC118	*upf2Δ upf3-1*	0.58

The mRNA abundances of the *CYH2* precursor and mRNA were determined in the strains shown and as described in Materials and methods. The abundances of the *CYH2* precursor and mRNA in the various strains were quantitated, and their ratios were determined.

Laird-Offringa 1992; Aharon and Schneider 1993; Peltz et al. 1993; Schiavi et al. 1994).

Studies on the nonsense-mediated mRNA decay pathway have been particularly fruitful in the identification of genes whose products are involved in mRNA turnover. In the yeast *S. cerevisiae* the products from the *UPF1*, *UPF2* (*SUA1*) and *UPF3* (*SUA6*) genes are involved in controlling the abundance of nonsense-containing mRNAs (Culbertson et al. 1980; Leeds et al. 1991, 1992; Pinto et al. 1992; Peltz et al. 1993; results presented here). The *UPF1*, *UPF2*, and *UPF3* genes elevate the concentration of nonsense-containing mRNAs in cells by increasing their half-lives (Leeds et al. 1991, 1992; Peltz et al. 1993; results presented here). Mutations in the *UPF1* gene have been identified and characterized (Leeds et al. 1991, 1992; Peltz et al. 1993), the identification and characterization of the *UPF2* gene is described here, whereas the *UPF3* (*SUA6*) gene has not yet been characterized. Furthermore, in *Caenorhabditis elegans*, seven *smg* alleles identified as extragenic suppressors of myosin heavy-chain B mutations increase the abundance of nonsense-containing myosin transcripts while not affecting the abundance of wild-type mRNAs (Hodgkin et al. 1989; Pulak and Anderson 1993). At present, the cloning of the *smg* genes has not been reported.

Sequencing of the *UPF2* gene and characterization of its 3600-nucleotide transcript suggest that it encodes a protein with a predicted molecular mass of 126.7 kD (Fig. 4). The polypeptide sequence located at its carboxyl terminus has a long stretch of acidic amino acids consisting of aspartic acid and glutamic acid repeats similar to amino acid sequences found in the nucleolin, nucleolar phosphoprotein B23, as well as nucleolar transcription factor UBF (Fig. 4; the homologous region is shown). These proteins are thought to be involved in ribosomal biogenesis, and their acidic regions contain phosphorylation sites that are demonstrated to be important for their functions. It suggests that the acidic amino acid rich region near the carboxyl terminus of Upf2p might be involved in protein–protein interactions modulated by phosphorylation.

As far as we can determine, *upf2Δ* strains are phenotypically identical to strains harboring the *upf1Δ* allele. *upf1Δ* strain and *upf2Δ* strains have the following similar characteristics: (1) The stabilities of nonsense-containing transcripts are increased in these strains compared with wild-type cells; (2) the mRNA decay rates of wild-type mRNAs are, for the most part, unaffected in these strains; (3) *upf1Δ* and *upf2Δ* strains are both slightly sensitive to the translation elongation inhibitor cycloheximide, whereas they do not show any sensitivities to paromomycin, a drug that decreases translational fidelity during elongation; (4) neither the *UPF1* or the *UPF2* gene is essential for vegetative growth under the growth conditions used, because haploid cells harboring either *upf1Δ* or *upf2Δ* alleles were viable with no apparent growth defect; (5) both *upf1Δ* and *upf2Δ* alleles can function as omnipotent suppressors (Leeds et al. 1992; Table 2, item 2). Furthermore, strains harboring both *upf1Δ* and *upf2Δ* alleles or *upf2Δ* and *upf3-1* were also viable with no apparent growth defect and did not further alter the abundance of the *CYH2* precursor when compared with each of the individual *upf* alleles. Taken together, these results suggest that *UPF1*, *UPF2*, and *UPF3* are involved in the same pathway.

The *sua* alleles were identified previously by selection of suppressors of a *cyc1-362* mutation, a mutation that results in an out-of-frame ATG codon 5′-proximal to the normal CYC1 translation start site (Stiles et al.1981). This mutation yields a *cyc1* allele that results in premature translation termination and should lead to the accelerated decay of this mRNA. Thus, it is anticipated that a subclass of the *sua* suppressors (Hampsey et al. 1991; Pinto et al. 1992a,b) might function by inactivating the nonsense-mediated mRNA decay pathway. The results described here demonstrate that *sua1* and *sua6* alleles most likely suppress the *cyc1-362* mutation by stabilizing their mRNAs (Fig. 6). Furthermore, we have demonstrated that *UPF2* and *SUA1* encode the same gene, whereas *SUA6* is the same gene as *UPF3*.

Remarkably, the *UPF2* gene has been isolated recently and shown to be involved in nonsense-mediated mRNA decay by a totally different approach than that described here (He and Jacobson, this issue). Utilizing a genetic system to detect protein–protein interactions in vivo (Fields and Song 1989), a search of putative interacting domains with the *UPF1* gene product has identified eight genes, called NMDs (for nonsense-mediated mRNA decay) that putatively interact with the Upf1 protein (for review, see Peltz et al. 1994). Deletion of the *NMD2* gene from the yeast genome was subsequently demonstrated to stabilize nonsense-containing mRNAs without affecting the decay of wild-type transcripts (F. He and A. Jacobson, pers. comm.). Comparison of the DNA restriction maps and the DNA sequences of the *UPF2* and *NMD2* genes revealed that they are the same gene. This result suggests that the products of *UPF1* and *UPF2* (*NMD2*) genes interact. Our present objective is to determine how the factors that are involved in the nonsense-mediated mRNA decay pathway function to accelerate the decay of nonsense-containing transcripts.

Materials and methods

Strains, media, and general methods

The yeast strains used in this study are listed in Table 1. The *E. coli* DH5α strain was used to amplify plasmid DNA. Yeast media was prepared as described (Rose et al. 1990). Yeast transformations were performed by the lithium acetate method (Schiestl and Gietz 1989). Tetrad analysis was performed as described (Rose et al. 1990).

Materials

Restriction enzymes were obtained from Boehringer Mannheim, New England Biolabs, and BRL. Radioactive nucleotides were obtained from either NEN ([γ-^{32}P]ATP) or Amersham ([α-^{32}P]dCTP). Oligonucleotides used in these studies were purchased from the UMDNJ-RWJ DNA synthesis center.

Isolation and characterization of the UPF2 *gene*

The plasmids pYCp50 (Ausubel et al. 1992), pYCplac33, and pYCplac112 (Gietz and Sugino 1988) were used in these studies. The *UPF2* gene was cloned from a pYCp50 yeast genomic library (purchased from ATCC) that was prepared from a partial *Sau*3A digest. Strain PLY136 was transformed with this library and a total of 5000 Ura$^+$ transformants were screened by replica-plating onto minimal media lacking uracil and histidine and grown at either 30 or 37°C for 4–5 days. Colonies that grew at 30°C but not at 37°C on minimal media lacking uracil and histidine were retested and nine strains harboring plasmids were isolated (YPF2-1 to YPF2-9). To confirm that the growth phenotype of the *upf2*$^-$ strains harboring plasmids was a consequence of the plasmids, a 5-FOA selection for the plasmids loss was performed (Rose et al. 1990). The plasmid pYCpA5 was isolated from strain YPF2-5 and propagated in *E. coli*.

Subcloning of the UPF2 *gene*

A restriction map of the yeast genomic DNA fragment in pYCpA5 was prepared (Fig. 3A). Plasmid pYCpAΔB is a derivative of pYCpA5 in which the *Bam*HI DNA fragment was deleted (Fig. 3B). This plasmid was constructed by cleaving of pYCpA5 with the enzyme *Bam*HI, isolation of the 9.9-kb DNA fragment, ligation, and amplification in *E. coli*. The following subclones of the yeast genomic DNA fragment in pYCpA5 were prepared by isolation of various DNA fragments and insertion of them into the yeast centromere plasmid pYCplac33 (see restriction map of the yeast genomic DNA fragment Fig. 3A): pYCpA3.5 (3.5-kb *Bam*HI–*Bam*HI DNA fragment), pYCpA5.0 (5.0-kb *Asp*718–*Eco*RI DNA fragment), pYCpAX6.6 (6.6-kb *Xba*I–*Xba*I DNA fragment), pYCpAp7.1 (7.1-kb *Asp*718–*Xho*I DNA fragment), and pYCpAB5.4 (5.4-kb *Bst*XI–*Xho*I DNA fragment). The multicopy plasmid pYEpUPF2 was constructed by isolation of the 7.1-kb *Asp*718–*Xho*I DNA fragment from plasmid pYCpA5 and insertion of this fragment into pYEplac112 (Fig. 3). This plasmid was transformed into the *upf2*Δ strain YGC112 and strain PLY18 and used in the analysis of the *UPF2* transcript. pPUCA5.0 was constructed by isolation of the *Asp*718–*Eco*RI DNA fragment from pYCpA5.0 (Fig. 3A) and insertion of it into pPUC18. The GenBank accession number for the sequence of the *UPF2* gene is U12137.

Preparation of the UPF2 *knockout allele*

pKOF2 was prepared to delete the *UPF2* gene from the yeast chromosome. pKOF2 was prepared by cleaving pPUCA5.0 with the restriction enzyme *Cla*I and replacing the 2.3-kb of the *UPF2* gene (nucleotide −1076 to 1288 containing UPF2 transcription initiation site and part of *UPF2* coding region; see Figs. 3A,B and Fig. 4) with a 1.57-kb DNA fragment harboring the *URA3* gene.

Preparation of a UPF1 *knockout allele*

pKOM was prepared to delete the *UPF1* gene from the yeast chromosome. First, pPUC19-UPF1 was constructed by insertion of the 4.2-kb *Eco*RI–*Bam*HI DNA fragment harboring the *UPF1* gene (Leeds et al. 1992) into pPUC19. Plasmid pKOM was then prepared by cleavage of pPUC19-UPF1 with *Mun*I and *Bst*XI and replacement of this 2.9-kb DNA fragment (base pairs 494–3426 in the *UPF1* gene map; Leeds et al. 1992) with a DNA fragment harboring the *URA3* gene imbedded between two HisG cassettes (Alani et al. 1987).

Preparation of a strain harboring genomic disruption of UPF1

Plasmid pKOM was digested with *Bam*HI and *Eco*RI, the 4.6-kb DNA fragment harboring the *upf1::HisG-URA3-HisG* disruption was transformed into strain RY262 (Table 1), and cells harboring the *UPF1* disruption were selected by plating on medium lacking uracil. The selected Ura$^+$ cells were then grown on medium containing 5-FOA to select for strains that lost the *URA3* gene as a consequence of recombination between the HisG cassettes. The deletion of the *UPF1* gene from the yeast chromosome was confirmed by DNA blotting analysis of *Bam*HI/*Eco*RI-digested genomic DNA. A radioactively labeled DNA fragment from the flanking sequences of the *UPF1* gene was used as the probe. The results of the Southern blotting analysis confirmed that the *UPF1* gene was deleted from the yeast chromosome (data not shown).

Preparation of a strain harboring genomic disruption of UPF2

Plasmid pKOF2 was digested with *Asp*718 and *Eco*RI and the 5-kb DNA fragment harboring the *upf2::URA3* disruption was introduced into the yeast strains RY262, Y52$^-$, PLY36, PLY139, and PLY18 (see Table. 1), and transformants were selected on medium lacking uracil. Deletion of the *UPF2* gene from the yeast chromosome was confirmed by Southern blotting of *Asp*718/*Eco*RI-digested genomic DNA as described above. A radioactively labeled 1.7-kb *Bam*HI–*Eco*RI DNA fragment containing the *UPF2* gene-coding region was used as a probe. The results of this analysis confirmed that the *UPF2* gene was deleted from the yeast chromosome (data not shown).

mRNA decay measurements, RNA preparation, and RNA analysis

mRNA decay rates were determined as follows: Cells were grown to mid-log phase (OD_{600} = 0.7–1.0) at 24°C, centrifuged, resuspended in 18 ml of the same medium, and incubated at 24°C for 10 min. Transcription was inhibited by thermal inactivation of RNA polymerase II by shifting the concentrated culture to 36°C by addition of 18 ml of medium preheated to 54°C. After the temperature shift, the culture was maintained at 36°C and aliquots (4 ml) were removed at various times. Upon removal of an aliquot, cells were collected by rapid centrifugation, the supernatants were removed by aspiration, and the cell pellets were frozen quickly in dry ice. Routinely, cells were frozen within 15 sec after removal of the culture aliquot. Total yeast RNA was isolated as described previously (Herrick et al. 1990; Parker et al. 1991). Equal amounts (usually 20–40 μg) of total

RNA from each time point of an experiment were analyzed by RNA blotting (Thomas 1980). Gels were stained with ethidium bromide before and after blotting to assess the efficiency of RNA transfer and to confirm the equal loading of RNA. Hybridizations with probes prepared by random priming (see below) were performed as described previously (Herrick et al. 1990). RNA blots were quantitated by use of a Bio-Rad model G-250 Molecular Imager or model G-670 Imaging Densitometer. Data were expressed as the $\log_{10}$ of the percentage of each RNA remaining versus time at 36°C. Reproducibility of mRNA decay rate measurements was ±15%.

Analysis of the UPF2 *transcript*

Total RNA was extracted from yeast strains PLY136 harboring plasmid pYCpAp7.1, strain PLY18 or YGC112 harboring plasmid pYEpUPF2, and strain YGC112 and RL92 without plasmid (Table 1). The strain RL92 harboring the *prp2* allele, which inhibits mRNA splicing at 37°C, was grown at 24°C and then shifted to 37°C for 6 hr. The splicing defect in *prp2* at 37°C was confirmed by testing the *CYH2* RNA product (data not shown).

Northern blotting analysis of the *UPF2* transcript was performed with RNAs isolated from the strains described above. Primer extension was performed as follows: 50 μg of total yeast RNA from strains harboring the plasmid pYEpUPF2 was used as the template for reverse transcription (end-labeled primer: 5′-GCTCGGGTGTTCAAATCATGCAAT-3′). The products of the reverse transcription were analyzed by electrophoresing on a sequencing gel. The location of the intron–exon junction was determined by a polymerase chain reaction (PCR; Brill and Stillman 1991). Briefly, 30 μg of total yeast RNA was reverse transcribed with MoMLV-RT and random hexamers as primers. An internal control consisting of the reaction mixture without MoMLV-RT was also performed at the same time. The reverse transcription product was precipitated with 0.77 M $NaClO_4$ and isopropanol and resuspended in 50 μl of H_2O. The product from the reverse transcription reaction (10 μl) was used as a template for PCR with the following primers: (a) 5′-TTAGGGCATGAGGATGAT-3′; (b) 5′-GGACAGAAATTATGGACG-3′; and (c) 5′-ATGTCAACAGAGGGGTTC-3′. The conditions for the PCR were 94°C, 5 min and then 94°C, 0.5 min, 50°C, 1 min, and 72°C, 1 min for 30 cycles. The products were run on a 1.5% agarose gel and a 390-bp DNA fragment (corresponding to the reverse transcription product from the spliced *UPF2* mRNA) (Fig. 5B) and a 500-bp DNA fragment (corresponding to the genomic and unspliced RNA) were isolated and the sequence of the fragments was determined by cycling sequencing (BRL; the conditions were 15 ng of DNA for the 500-bp fragment and 18 ng of DNA for two 390-bp fragments with the primer 5′-GGGAAAGACTTCTTCGCCATTCC-3′). PCR conditions were 95°C, 3 min and then 95°C, 0.5 min, 55°C, 0.5 min, and 72°C, 1 min for 20 cycles. For the next 10 cycles the conditions were 95°C, 0.5 min and 70°C, 1 min.

Drug sensitivity assay

RY262 derivative strains Y52$^-$ containing pYCplac33, YGC114, YGC116, and wild-type RY262 containing pYcplac33 (Table 1) were grown to saturation in medium lacking uracil. The cells were diluted to OD_{600} = 0.4–0.6, and 300-μl aliquots were plated on medium lacking uracil. A 0.25-inch-diam. disc was placed on the plate. Either 10 μl of paromomycine sulfate (250 μg/μl; Sigma) or cycloheximide (0.25 μg/μl; Sigma) was aliquoted onto the disc. The plates were incubated at 24°C for 3 days, and the drug sensitivities of the cells were determined by measurement of the diameter of the zone of growth inhibition.

Preparation of radioactive probes

DNA probes were labeled to high specific activity with [α-^{32}P]dCTP (Feinberg and Vogelstein 1983) or by 5′-end labeling of single-stranded oligodeoxynucleotides with [γ-^{32}P]ATP (Sambrook et al. 1989). A 1.7-kb *Bam*HI–*Eco*RI fragment was used as hybridization probe to monitor the *UPF2* transcript. The other radioactive probes used to monitor the decay of mRNAs were the following: a 0.6-kb *Eco*RI–*Hin*dIII fragment from the *CYH2* gene; a 4-kb *Sph*I–*Sac*I fragment from the *HIS4* gene; a 1.5-kb *Sal*I–*Bst*EII fragment from the *LEU2* gene; a 1.6-kb *Eco*RV–*Hin*dIII fragment from *MATα1* gene; and a 5-kb *Hin*dIII–*Hin*dIII fragment from the *TIF4631* gene were radiolabeled by random priming.

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Identification of an additional gene required for eukaryotic nonsense mRNA turnover

(*Saccharomyces cerevisiae*/mRNA decay/translation/gene expression)

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ABSTRACT **Loss of function of any one of three *UPF* genes prevents the accelerated decay of nonsense mRNAs in *Saccharomyces cerevisiae*. We report the identification and DNA sequence of *UPF3*, which is present in one nonessential copy on chromosome VII. Upf3 contains three putative nuclear localization signal sequences, suggesting that it may be located in a different compartment than the cytoplasmic Upf1 protein. Epitope-tagged Upf3 (FLAG-Upf3) does not cofractionate with polyribosomes or 80S ribosomal particles. Double disruptions of *UPF1* and *UPF3* affect nonsense mRNA decay in a manner indistinguishable from single disruptions. These results suggest that the Upf proteins perform related functions in a common pathway.**

Several genes have been identified in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* that are required for the accelerated rate of decay that occurs when translation terminates prematurely because of frameshift or nonsense mutation (1–5). Nonsense mRNA decay has been observed in a wide range of eukaryotic organisms and may contribute to the etiology of disease processes in humans. A form of β-thalassemia common in human Mediterranean populations has been shown to result from an amber (UAG) nonsense mutation that reduces β-globin mRNA accumulation and may exacerbate the symptoms of the disease (6). The effects of nonsense mutations that arise in somatic cells could also be exacerbated because rapid decay ensures complete loss of function of a mRNA that might otherwise produce some functional product (3).

In *S. cerevisiae*, mutations in *UPF1, UPF2*, and *UPF3* prevent nonsense mRNA decay (1, 2, 4, 5). They were isolated as allosuppressors of *his4-38*, a +1 frameshift mutation in the *HIS4* gene that causes premature translational termination (7). *UPF1* codes for a 109-kDa protein that contains putative RNA binding domains, suggesting the potential for direct interaction with mRNA (2). *UPF1* behaves like a soluble factor that associates with polyribosomes, but is much less abundant than individual ribosomes (8). *UPF2* codes for a 126-kDa protein that functions in the cytoplasm (4, 5). The *UPF2* gene was identified among clones retrieved by a two-hybrid screen using *UPF1* DNA as bait, indicating that the Upf1 and Upf2 proteins may interact physically (4).

To understand how Upf3 might be related to Upf1 and Upf2, we have cloned the *UPF3* gene, determined the DNA sequence,† and shown that the gene product is not essential for growth. Phenotypic analyses of single and double mutants suggest that both genes may be required in the same pathway.

MATERIALS AND METHODS

Strains, Plasmids, Genetic Techniques, and Media. The following strains of *S. cerevisiae* were used: PLY100 (*MATa ura3-52 trp1-7 leu2-3,-112*), PLY107 (*MATα his4-38 SUF1-1 ura3-52 leu2 trp1-Δ1 lys1-1*), PLY140 (*MATa his4-38 SUF1-1 upf3-1 trp1-1 ura3-52 trp1-1*), BSY12 (*MATα his4-38 SUF1-1 upf3-1 ura3-52 trp1-1*), BSY202 (*MATα his4-38 upf3-1 ura3-52 leu2-2 trp1⁻ rpb1-1*), BSY1001 (*MATα trp1-Δ1 his4-38 SUF1-1 upf3-Δ1 ura3-52 lys1-1 leu2*), BSY1044 (*MATa ura3-52 trp1-7 leu2-3,-112 upf3-Δ2*), BSY1077 (*MATa ura3-52 leu2-3,-112 trp1-7 upf3-Δ2 esp1-1*), BSY1088 (*MATα ura3-52 leu2-3 ade6*), BSY2103 (*MATa ura3-52 trp1-7 leu2-3,-112, upf3-Δ2 rpb1-1* [YRpPL81]), BSY2111 (*MATα ura3-52 upf3-Δ2 trp1 leu2-3,-112*), BSY2015 (*MATa ura3-52 trp1-7 leu2-3,-112 upf3-Δ2* [YRpPL81]), BSY2115 (*MATα ura3-52 upf1-Δ1 upf3-Δ1 trp1 his4-38 leu2-3,-112 lys1-1 rpb1-1*), and BSY2116 (*MATa ura3-52 upf1-Δ1 upf3-Δ1 trp1 leu2-3,-112 lys1-1 rpb1-1*). Isogenic strains were used that differ only by the absence (−) or presence (+) of extrachromosomal *UPF3*.

The following plasmids were used: YCpBSL1 (*CEN4, URA3*, and a *Sau3*AI–*Sau3*AI insert carrying *UPF3*), YCpBSL2 (*CEN4, URA3*, and a *Cla* I–*Sau3*AI insert carrying *UPF3*), YEpBSL2 (2-μm plasmid origin, *URA3, Bam*HI–*Sal* I insert containing *UPF3*), pBSL1 (pUC19 containing a *Bam*HI–*Sal* I insert carrying *UPF3*), YCpBSL4 (*CEN4, TRP1, Bam*HI–*Sal* I insert containing *UPF3*), YEp[F]BSL2 (2-μm plasmid origin, *URA3*, and a *Bam*HI–*Sal* I insert carrying *FLAG-UPF3*), YCp[F]BSL4 (same as YCpBSL4 except that it carries *FLAG-UPF3* instead of *UPF3*), pBSL12 (pUC18 containing a *Bgl* II–*Hin*fI insert carrying *upf3-Δ1*), pBSL321 (pUC18 containing a *Bgl* II–*Hin*fI insert carrying *upf3-Δ2*), and YRpPL81 (*TRP1, ARS1*, and a *his4-38*, -UAA–*lacZ* gene fusion; see Fig. 4*A*).

Standard genetic methods and media for yeast were used (9, 10). Yeast transformation was by the method of Ito *et al.* (11). Standard bacterial methods and media were used (12).

Nucleic Acid Methods. Yeast chromosomal DNA was prepared by the method of Hoffman and Winston (13). Plasmid DNA isolation and DNA and RNA blotting were performed as described (14). To determine gene copy number, Southern hybridization was performed in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7)/0.5% SDS at 65°C. The filter was washed in 1× SSC/0.1% SDS for 30 min at room temperature and then for 60 min at 65°C. The DNA sequence was determined by the method of Sanger *et al.* (15). mRNA half-lives were determined by the temperature-shift method for blocking transcription in strains carrying *rpb1-1* (1).

Allosuppression. The ability of *upf3* alleles to confer allosuppression of *his4-38* in the presence of the tRNA frameshift suppressor *SUF1-1* has been described (1, 2). Growth was assayed by using synthetic dextrose medium lacking histidine (SD−His) at 37°C in strains carrying *his4-38* and *SUF1-1*. In this assay, *UPF3* confers lack of growth, whereas mutations that cause loss of *UPF3* function allow growth.

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Abbreviations: NLS, nuclear localization signal; ORF, open reading frame.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession number L41153).

Polyribosome Analysis. Polyribosomes were fractionated on 12 ml of 15–50% continuous sucrose gradients (1, 16, 17). RNA was extracted from the fractions and analyzed by Northern blotting using an *Escherichia coli lacZ* probe made from a 2.4-kb *Cla* I–*Bam*HI fragment of the *lacZ* gene. Data are expressed as a percentage of total counts derived by quantitative determination of radioactivity in each band normalized to total counts across all fractions.

Immunodetection. The FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was added to the N-terminus of Upf3 by inserting DNA that codes for FLAG after the first ATG in *UPF3* by inverse PCR (18). The reaction was primed with 40- and 45-nucleotide oligomers that contain fused FLAG and 5′ *UPF3* sequences that bracket the translation initiation codon. Anti-FLAG antibodies were from IBI (Kodak). Protein extraction and immunoblotting are as described (19).

RESULTS

Loss of *UPF3* Function Inactivates Nonsense mRNA Decay. HIS4 mRNA has a half-life of 18–20 min (1, 2). The mRNA encoded by *his4-38* contains a +1 frameshift that causes premature termination at a UAA codon (7). In strain BSY202(+), which carries *UPF3*, the his4-38 mRNA half-life is between 2 and 3 min (Fig. 1*A*). In strain BSY202(−), which carries *upf3-1*, the his4-38 mRNA half-life increases to ≈12 min. These results indicate that *UPF3* is required for rapid decay of frameshift and nonsense mRNAs.

We tested other nonsense mutations to see if they were suppressed by *upf3-1* and by *upf3*-null mutations described below. In addition to suppression of *leu2-2* (UGA), *his4-166* (UGA), and *leu2-1* (UAA) (reported previously; ref. 2), we found that *tyr7-1* (UAG) and *met8-1* (UAG) are also suppressed. For all suppressible *his4* and *leu2* alleles, suppression has been shown to result from a change in the half-life and accumulation of mRNA (refs. 1 and 2 and this paper). Some mutations were not suppressed, including *ade2-1* (UAG) and *his4-713* (+1C). The premature stop codon in *his4-713* is near the 3′ end of the *HIS4* coding region (7). Like many other 3′-proximal nonsense mutations (20), *his4-713* does not affect the turnover rate (1).

Analysis of a cross between strains PLY140 and PLY107 shows that the changes in nonsense mRNA accumulation are linked to *upf3-1* (Fig. 1 *B* and *C*). The segregation of *upf3-1* was followed by using allosuppression of his4-38 mRNA in the presence of the tRNA frameshift suppressor *SUF1-1*. In each meiotic tetrad, two spores were His^+ (*upf3-1*) and two were His^- (*UPF3*) at 37°C (Fig. 1*C*). mRNA levels were determined by Northern blotting of total RNA from strains PLY140 and PLY107 and from all four spores of four tetrads. As shown for one tetrad (Fig. 1 *B* and *C*), the two spores that grew at 37°C on SD−His medium had significantly higher levels of mRNA than the two spores that failed to grow. Growth at 37°C cosegregated with increased mRNA abundance in all four tetrads.

The *UPF3* Gene Codes for a 44.9-kDa Protein. The *UPF3* gene was cloned by screening a yeast genomic library for plasmids that complement the recessive *upf3-1* mutation in strain BSY12. A plasmid called YCpBSL1 was rescued from one transformant into *E. coli* by selecting for ampicillin resistance. YCpBSL1 contains a 10-kb yeast genomic DNA insert. A 2.7-kb *Bam*HI–*Sal* I fragment that complements *upf3-1* was used to determine the DNA sequence (Fig. 2 *A* and *C*). A single open reading frame (ORF) of 1161 bp was found that lacked the TACTAAC sequence indicative of an intron. The predicted product is a 44.9-kDa protein of 387 amino acids. Three regions of the amino acid sequence contain basic arginine/lysine-rich stretches that resemble bipartite nuclear localization signal (NLS) sequences found in nucleoplasmin and other proteins that are targeted to the nucleus (21). Two are located near the N terminus at amino acids 15–31 and 58–74 and contain the sequences Lys-Lys-Xaa_{10}-Arg-Gly-Lys-Ser-Lys and Arg-Arg-Xaa_{10}-Asn-Tyr-Lys-Arg-Lys, respectively. A third is located near the C terminus at amino acids 284–300 and contains the sequence Lys-Lys-Xaa_{10}-Pro-Lys-Lys-Lys-Arg.

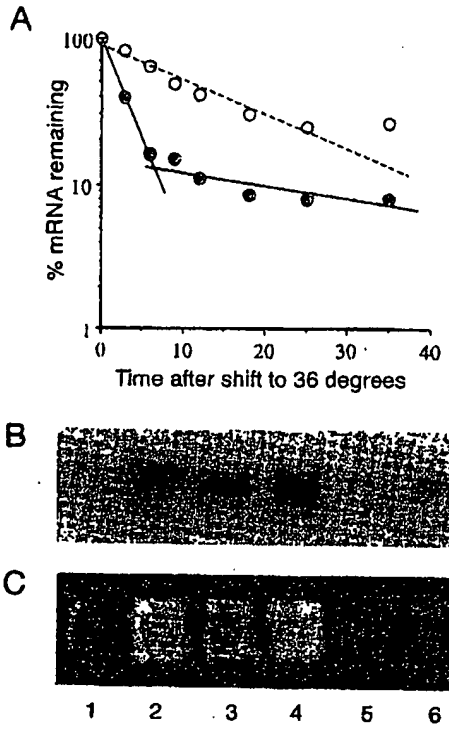

FIG. 1. Effect of *upf3-1* on nonsense mRNA decay. (*A*) The half-life of his4-38 mRNA was determined in strains BSY202(+) (*UPF3*) (●) and BSY202(−) (*upf3-1*) (○). The half-lives were determined by quantitative Northern hybridization analysis at 0, 3, 6, 9, 12, 18, 25, and 35 min following the termination of transcription that occurs when *rpb1-1* strains are shifted to 36°C (1, 2). The decay rate was calculated from the plot of percent RNA remaining vs. time; data points within the first phase of decay were used. It has not been determined whether the second apparent decay phase is of functional significance or whether it is caused by residual transcription due to leakiness of *rpb1-1*. (*B*) Northern blot (2) showing the relative accumulation of his4-38 mRNA in strains PLY107 (lane 1), PLY140 (lane 2), and the four spores of a tetrad derived from a PLY140 × PLY107 cross (lanes 3–6). The blot was probed with *ACT1* DNA (actin) to control for loading differences. Actin mRNA levels were the same in all spores (data not shown). (*C*) Growth rates of the strains analyzed in *B* were compared after 2 days of incubation at 37°C on SD−His medium.

FLAG-UPF3, which codes for an epitope-tagged allele of *UPF3*, was analyzed to estimate the size of the gene product. In the allosuppression assay, growth at 37°C on SD−His/−Ura medium was inhibited to the same extent in strains carrying either *FLAG-UPF3* or *UPF3*, indicating that the FLAG-Upf3 protein is functional. The multicopy plasmids YEp[F]BSL2 and YEpBSL2, containing *FLAG-UPF3* and *UPF3*, respectively, were transformed separately into strain BSY1001. A Western blot (Fig. 3*A*) shows that FLAG-Upf3 was detected in the region of the gel where 45- to 50-kDa proteins migrate. Using differential cellular fractionation, we also detected FLAG-Upf3 in a derivative of strain BSY2111 that carries the *FLAG-UPF3* gene on a single-copy centromeric plasmid (Fig. 3*B*). FLAG-Upf3 was found primarily in the sedimentable fraction after a 20-min centrifugation at 12,130 × *g* in 0.15 M NaCl. FLAG-Upf3 was solubilized from the pellet by extracted in 1 M NaCl. In this procedure, polyribosomes and 80S ribosomal particles are located in the nonsedimentable fraction (not shown) and therefore do not cofractionate with FLAG-Upf3.

***UPF3* Is a Single-Copy, Nonessential Gene on Chromosome VII.** The *UPF3* copy number was determined by genomic Southern blotting with a ^{32}P-labeled 1.59-kb *Nde* I–*Hin*I restriction fragment containing the *UPF3* ORF plus 5′ and 3′

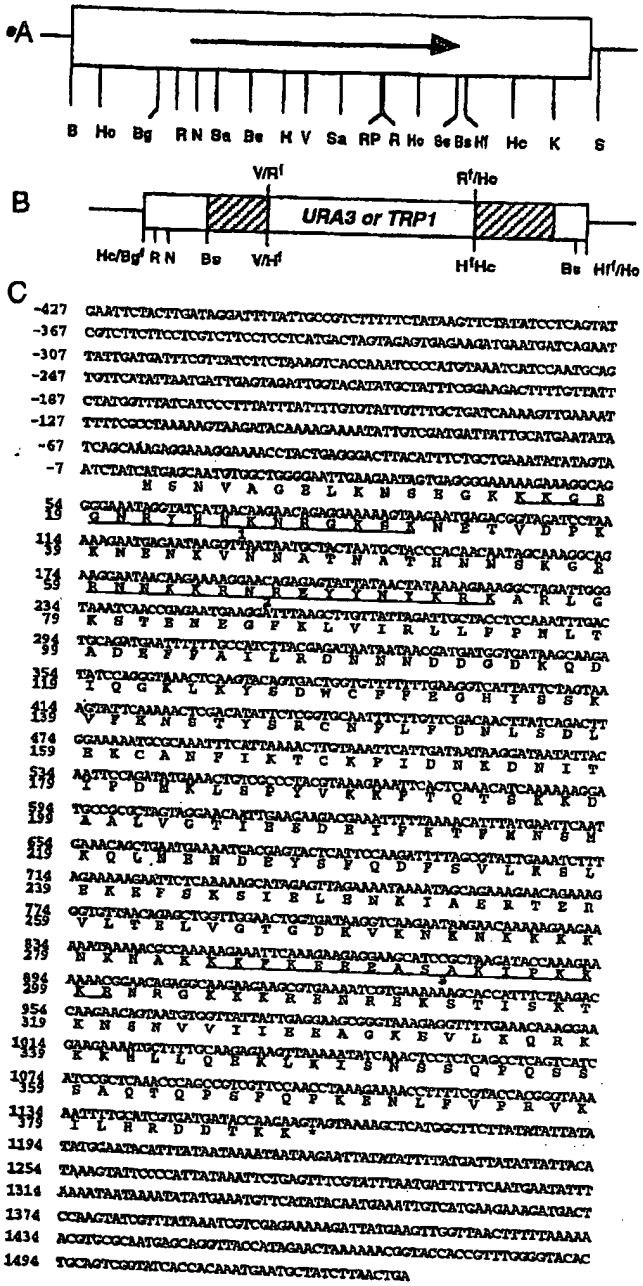

FIG. 2. (*A*) Restriction map of the 2.7-kb *Bam*HI–*Sal* I fragment carrying *UPF3*. The open box denotes yeast genomic DNA inserted in the vector (solid lines). The arrow indicates the position and direction of transcription of the *UPF3* gene. Restriction sites are as follows: B, *Bam*HI; Hc, *Hin*cII; Bg, *Bgl* II; R, *Eco*RI; N, *Nde* I; Ss, *Ssp* I; Bs, *Bsp*HI; H, *Hin*dIII; V, *Eco*RV; P, *Pvu* II, Hf, *Hin*fI; K, *Kpn* I, and S, *Sal* I. (*B*) Structure of *upf3-Δ1* and *upf3-Δ2*, which carry *TRP1* and *URA3* insertions, respectively. A slash denotes a blunt-end ligation. V/R^f and R^f/Hc show the junctions of the *TRP1* insertion. V/H^f and H^f/Hc show the junctions of the *URA3* insertion. Bgf, Hf^f, R^f, and H^f denote the restriction sites corresponding to *Bgl* II, *Hin*fI, *Eco*RI, and *Hin*dIII that were blunt-ended by the Klenow fragment. Hatched areas indicate the locations of the *UPF3* coding sequences. (*C*) DNA sequences of the *UPF3* gene and the corresponding amino acid sequence. The A of the first ATG in the ORF is designated the +1 nucleotide. The three regions that match the consensus for the bipartite NLS sequence are underlined and labeled 1, 2, and 3.

flanking DNA. Using stringent hybridization and washing conditions, we observed two bands when genomic DNA was digested with the restriction enzymes *Hin*dIII or *Pvu* II. For *Bgl* II or *Kpn* I, one band was observed (data not shown). By

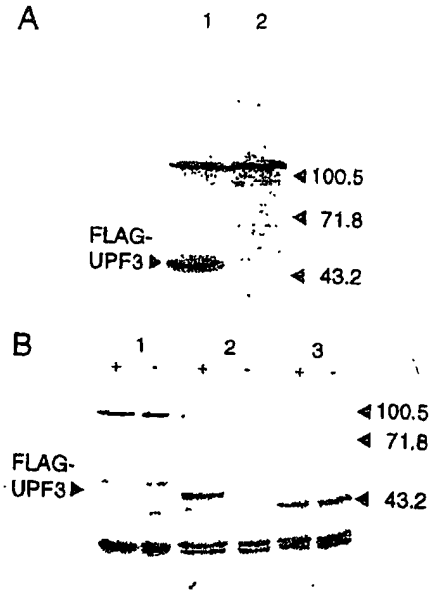

FIG. 3. Immunological detection of FLAG-Upf3. The Upf3 protein was tagged at the N terminus with the FLAG epitope. (*A*) Total protein extracts were prepared from strain BSY1001 separately transformed with the multicopy plasmids YEp[F]BSL2 (*FLAG-UPF3*) and YEpBSL2 (*UPF3*). Equal amounts of the protein extracts were loaded in lane 1 (FLAG-Upf3) and lane 2 (Upf3) and fractionated by SDS/7.5% PAGE. The proteins were analyzed by Western blotting with FLAG M2 monoclonal antibodies (mAbs). (*B*) BSY2111 transformants containing *FLAG-UPF3* on plasmid YCp[F]BSL4 (lanes +) and a control plasmid (YCpBSL4) that lacks *FLAG-UPF3* (lanes −) were fractionated as described (22). Fractions were separated by SDS/10% PAGE and analyzed with FLAG M2 mAbs as follows: supernatant from 12,130 × *g* centrifugation for 20 min in 0.15 M NaCl (lane 1), supernatant from a 1.0 M NaCl extraction of the sedimentable fraction (lane 2), and supernatant from a 2% deoxycholate extraction of the sedimentable fraction (lane 3). Sizes are in kDa.

comparing the position of each band with the position predicted from the restriction map (Fig. 2*A*), the results show that there is only one copy of the *UPF3* gene per haploid genome.

To examine the phenotype of complete loss of function, the *UPF3* gene was disrupted by replacing a 424-bp *Eco*RV–*Hin*cII fragment with either the *TRP1* or the *URA3* genes (*upf3-Δ1* and *upf3-Δ2*, respectively; Fig. 2*B*). The disruptions were introduced by gene replacement into strains BSY1001 and BSY1044. After gene disruption, both strains were viable, with no reduction in growth rate in YPD (yeast extract/peptone/dextrose) medium. When they were grown in SD−His medium, the extent of growth inhibition was similar to strains carrying *upf3-1*, indicating that *upf3-1* confers a null phenotype. Since there are no additional *UPF3* gene copies, the Upf3 protein is not essential for growth.

In tetrads from a cross between strains BSY1001 and PLY140, 21 of 21 segregated 2 Trp$^+$: 2 Trp$^-$ spores, indicating that the *upf3-Δ1* allele (*upf3*::*TRP1*) follows Mendelian segregation; 21 of 21 tetrads also segregated 4 His$^+$: 0 His$^-$ spores at 37°C on SD−His medium (parental ditype). Since no recombination between *upf3-Δ1* and *upf3-1* was detected, the two mutations are genetically linked. This verifies that the original clone is the *UPF3* gene.

The *UPF3* gene was mapped to chromosome VII by probing chromosomes separated on a CHEF (contour-clamped homogeneous electrophoretic field) gel with radioactively labeled YCpBSL2 plasmid DNA. An ordered array of bacteriophage λ–genomic yeast DNA clones (23) was then probed with a labeled 1.7-kb *Nde* I–*Kpn* I fragment from plasmid pBSL1. Results indicate that *upf3* is located between *CEN7* and *spt6* on the right arm of chromosome VII. We analyzed 110 tetrads

Table 1. Accumulation of his4-38 and HIS4 mRNA in single and double mutant strains carrying *upf1-Δ1* and *upf3-Δ1*

Strain	Transcript	Relative abundance* upf^-/UPF^+
BSY2115 (1^+, 3^-)	his4-38	3.2 ± 0.3
BSY2115 (1^-, 3^+)	his4-38	3.0 ± 0.2
BSY2115 (1^-, 3^-)	his4-38	2.9 ± 0.1
BSY2116 (1^+, 3^-)	HIS4	1.3
BSY2116 (1^-, 3^+)	HIS4	1.2
BSY2116 (1^-, 3^-)	HIS4	1.2

The *UPF1* and *UPF3* genes were introduced into strain BSY2115 (*his4-38 upf1-Δ1 upf3-Δ1*) and BSY2116 (*HIS4 upf1-Δ1 upf3-Δ1*) on multicopy plasmids. The nomenclature (1^+), (1^-), (3^+), and (3^-) denotes whether wild-type or mutant alleles of *UPF1* and *UPF3* are present in each strain.

*The relative abundance of his4-38 or HIS4 mRNA in each strain was determined by measuring mRNA accumulation (2) and comparing it with that observed in the isogenic strain carrying *UPF1* and *UPF3* genes on one plasmid. The mRNAs were detected by Northern blotting with a radiolabeled probe from the *HIS4* coding region. The extent of accumulation was determined by assaying radioactivity with a Betagen blot analyzer. Error bars are based on three repeat experiments. The blots were reprobed with ACT1 (actin) mDNA to standardize the amount of RNA loaded in each lane.

from a three-point cross (BSY1077 × BSY1088) heterozygous for the chromosome VII markers *upf3-Δ2* (scored at Ura^+), *ade6*, and *esp1*. Map distances were as follows: *upf3–ade6*, 9 centimorgans (cM) (90 parental ditype, 20 tetratype); *upf3–esp1*, 21.3 cM (63 parental ditype, 47 tetratype); and *ade6–esp1*, 33 cM (47 parental ditype, 2 nonparental ditype, 61 tetratype). The most likely gene order is *CEN7-ADE6-UPF3-ESP1*.

Accumulation of Nonsense mRNA in *upf1/upf3* Double Mutants. Northern blotting was used to examine his4-38 mRNA accumulation in a haploid double mutant carrying null mutations in both *UPF1* and *UPF3*. Strains were made genetically isogenic by transforming strain BSY2115 (*upf1-Δ1 upf3-Δ1*) with multicopy plasmids carrying the relevant wild-type *UPF* genes (Table 1). Single disruptions of *UPF1* or *UPF3* resulted in a 3-fold increase in his4-38 mRNA accumulation. Nearly identical results were obtained when both genes were simultaneously disrupted. When HIS4 mRNA accumulation was examined in a similar set of isogenic derivatives of strain BSY2116, we found that accumulation was unaffected by *UPF1* or *UPF3*. We conclude that the effects of loss of Upf1 and Upf3 function are nearly identical, nonadditive, and specific to mRNAs containing a premature stop codon.

Decay of Nonsense mRNA Produced from a *his4-lacZ* Gene Fusion. We assessed how loss of Upf3 function affects the decay of a *his4-lacZ* nonsense mRNA in which translation was previously shown to terminate efficiently (1) because of multiple premature stop codons in all three reading frames near the 5′ end of the fused *his4-lacZ* ORF (Fig. 4*A*). In the isogenic strains BSY2015(+)(*UPF3*) and BSY2015(−)(*upf3-Δ2*), both of which carry an integrated copy of the *his4-38,−UAA/lacZ* fusion, no β-galactosidase activity was detected by a qualitative assay (24) after 16 hr of development. This shows there is no translational readthrough into the *lacZ* coding region regardless of whether *UPF3* or *upf3-Δ2* is present.

The half-life of the fusion mRNA was measured in strains BSY2103(+) and BSY2103(−) (Fig. 4*B*) (for methods, see the legend to Fig. 1*A*). In BSY2103(+), the half-life of the 3.6-kb fusion mRNA is about 5 min compared with a half-life of 28 min in BSY2103(−). An 8.4-kb transcriptional readthrough product detected on the Northern blots exhibited a similar change in half-life (data not shown). This indicates that loss of *UPF3* function stabilizes the fusion mRNA. Loss of *UPF3* function had no effect on the half-life of a *HIS4-lacZ* fusion that contains an uninterrupted reading frame with no premature stop codons (data not shown).

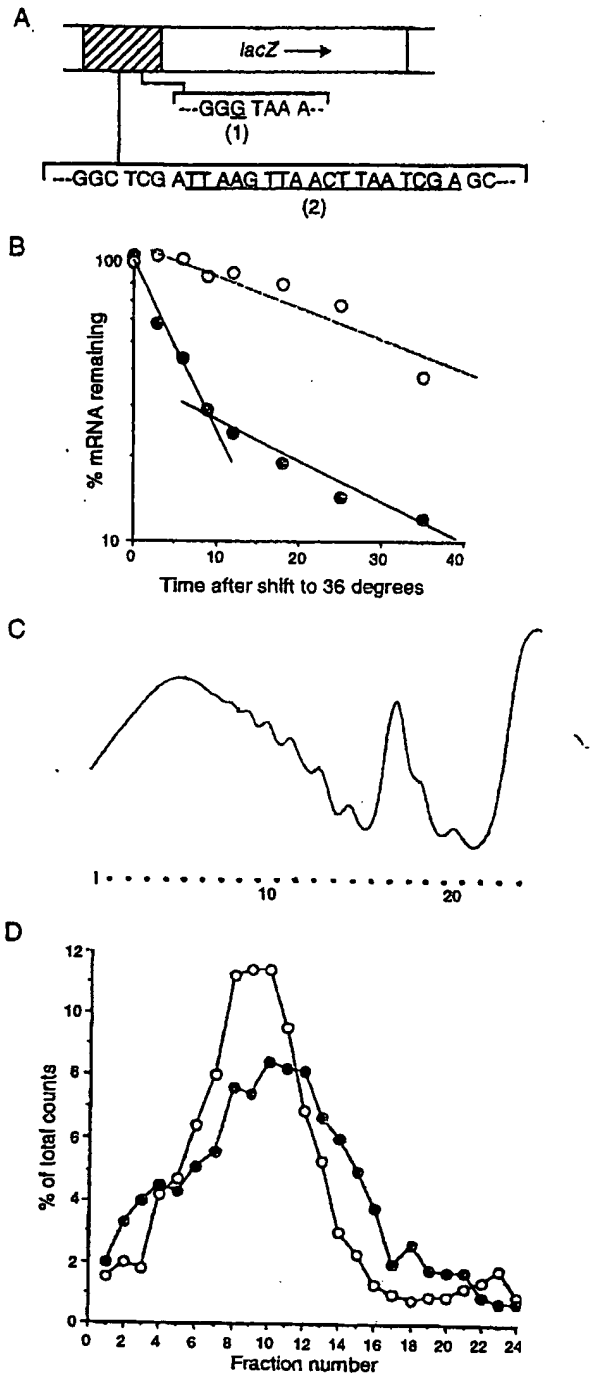

FIG. 4. Behavior of a nonsense mRNA that terminates translation efficiently. (*A*) Structure of *his4-38,−UAA/lacZ*. The *his4* region is hatched. The +1G insertion in *his4-38* is underlined and labeled (1). A linker insertion containing multiple stop codons in each reading frame is underlined and labeled (2). (*B*) The half-life of the fusion mRNA determined in strains BSY2103(+) (*UPF3*) (●) and BSY2103(−) (*upf3-Δ2*) (○). The half-lives were determined as described in Fig. 1*A*. (*C*) The distribution of polyribosome peaks in sucrose gradients was determined by monitoring the A_{260} absorption profile. The A_{260} profiles for the two strains were nearly identical. Only the profile for strain BSY2103(+) is shown. (*D*) Distribution of the 3.6-kb his4-38,−UAA/lacZ fusion mRNA determined by Northern blotting of fractions from the sucrose gradients in *C* using a 2.4-kb *Cla* I–*Bam*HI *lacZ* probe. RNA was extracted from strains BSY2103(+) (*UPF3*$^+$) (●) and BSY2103(−) (*upf3-Δ2*) (○). As a control, the blots were stripped and reanalyzed with an *ACT1* (actin) probe. Actin mRNA peaked in fractions containing larger polyribosomes than those corresponding to his4-38,−UAA/lacZ (not shown).

Polyribosomes isolated from strain BSY2103(+) and BSY2103(−) were fractionated by centrifugation through 15%–50% sucrose gradients (Fig. 4C). The distribution of the 3.6-kb fusion mRNA was determined by Northern blotting across the gradient (Fig. 4D). The fusion mRNA is distributed in a similar manner across fractions that contain polyribosomes regardless of the presence or absence of *UPF3* function.

DISCUSSION

The general pathway for mRNA decay in yeast involves a sequence of temporally ordered events, including the shortening of the poly(A) tail, removal of the 5′ cap, and exonucleolytic digestion in the 5′ to 3′ direction (25). The nonsense mRNA decay pathway shares common steps but has the unusual feature that the temporal requirement for poly(A) shortening is bypassed and the 5′ decapping reaction occurs in the presence of a long poly(A) tail (26). The Upf1, Upf2, and Upf3 proteins may in some way contribute, either directly or indirectly, to the decoupling of poly(A) tail structure from the remaining steps in the general decay pathway.

We recently established that Upf1 is cytoplasmic and is associated with actively translating polyribosomes (8). The Upf1 sequence contains several signature motifs that give clues to its function, including a cysteine-rich region that may bind zinc and an NTP-binding/RNA helicase-like domain, suggesting a potential for direct interaction with RNA (2). Since the Upf2 protein physically interacts with Upf1 (4), it appears likely that these two proteins are part of a complex that associates with polyribosomes. Upf2 has been reported to contain a bipartite NLS sequence, but overexpression of a UPF2 peptide fragment has been found to inhibit nonsense mRNA decay only when localized to the cytoplasm, indicating that at least one function of Upf2 is executed in the cytoplasm (4). This does not preclude the possibility that Upf2 resides in both the nucleus and the cytoplasm.

Mutations in *UPF3* have phenotypes similar to mutations in *UPF1* and *UPF2*. They suppress frameshift and nonsense mutations in a variety of genes and have similar effects on nonsense mRNA accumulation and decay (refs. 1, 2, 4, and 5 and this paper). We examined the translation and stability of a *his4-lacZ* fusion that produces a nonsense mRNA that terminates translation efficiently at sites upstream of the *lacZ* coding region. Upf1 (1) and Upf3 both promote decay of this nonsense mRNA, indicating that the increase in decay rate does not correlate with the extent of readthrough past a premature stop codon. Strains that are double null for *UPF1-UPF2* (5) or *UPF1-UPF3* have nonadditive effects on the accumulation of nonsense mRNA. Although other interpretations are possible, the most likely explanation of these phenotypes is that the products of *UPF* genes act in a common pathway leading to accelerated mRNA decay.

Like Upf2, the Upf3 polypeptide contains lysine/arginine-rich sequences that resemble the bipartite NLS sequence known to target proteins to the nucleus. Of all known proteins containing a bipartite NLS, 95% are targeted to the nucleus, whereas most of the remainder are secreted outside the cell or targeted to other organelles (27). Given that nuclear transport of mRNA and nonsense mRNA decay may be coupled, as proposed for animal cells (28), the functional significance of NLSs in *UPF2* and *UPF3* needs to be examined further. The finding that FLAG-Upf3 can be separated from polyribosomes and 80S ribosomal particles by differential cellular fractionation provides an additional incentive to determine the cellular location of Upf3. Unfortunately, the FLAG epitope proved unsuitable for immunolocalization. The intensity of background fluorescence made it impossible to distinguish the FLAG-Upf3 signal.

None of the known Upf proteins identified in yeast are essential for viability. Also, it was reported that mutations in the *smg* genes in *C. elegans*, whose products are required in nonsense mRNA decay in this organism, have some effects on development but are not lethal (3). Without knowing the exact functions of any of these genes, it seems likely that the nonsense mRNA decay pathway itself is dispensable for viability. Nonetheless, it may confer some advantages to eukaryotic organisms. It has been suggested that the pathway may serve to minimize the concentration of truncated polypeptides that accumulate through errors in gene expression, thereby reducing the chances that they could act in a deleterious fashion as poison subunits (29). The pathway also appears to control the expression of some natural mRNAs and might serve a second purpose in the regulation of specific genes (1).

Although the general effects of inactivation of the pathway on growth, viability, and development are subtle, it appears likely that nonsense mRNA decay influences the phenotypes of germ-line nonsense mutations found in the human population and may also influence the phenotypes of nonsense mutations that arise in somatic cells. All three known genes required for nonsense mRNA decay in yeast have now been cloned and characterized (refs. 1, 4, and 5 and this paper). Our efforts are currently focused on unraveling the mechanism of decay and identifying the natural mRNA targets, at which point the purpose of this pathway should become more clear.

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